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Characterisation of generation dependent variations in tenocytes for tissue engineering cell expansion applications

Kular, Jaspreet

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Characterisation of generation dependent variations in tenocytes for tissue engineering cell expansion applications

Jaspreet K. Kular

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Declaration of Authorship

This is all my own work except where I have indicated via references or other forms of acknowledgement.

Signature:

Date:

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Abstract

This work investigates the isolation, characterisation and use of adult and foetal tenocytes with PLGA (Polylactic-co-glycolide) using a hollow fibre bioreactor (HFB) system *in vitro* for tendon cell expansion applications for tissue engineering. Due to the poor healing mechanisms of adult tendon injuries and the high rate of complications that result from current surgical methods, there has long been a focus on regenerative medicine for an effective alternative treatment. It is well established that foetal tissue has the ability to undergo regenerative healing. Presently, adult tendon injuries are either treated using surgical or conservative methods. However, due to scar formation after the healing process, the full function of the tendon is never regained. Therefore, the ability to successfully grow tenocytes *in vitro* for use in clinical settings would be highly regarded.

Variations of two cell isolation methods, explant culture and enzyme dissociation were tested on adult (aged between 10-14 weeks) and foetal E15.5 (embryonic day 15.5) murine tendon tissue to extract tenocytes for subsequent cell culture. Whilst the foetal tenocytes could be extracted from the tissue with both methods, due to the inherently low cell population of adult tendon tissue, explant culture was a lengthy process for the adult tenocytes to be obtained. However, upon using 2ml blend of dispase (4mg/ml) and collagenase type IV (3mg/ml) enzymes to treat the tissue at 37 °C for 1 hour the tenocytes were released immediately from the tissue and available to culture. In addition to this, quantitative cell culture was made possible unlike explant culture, which is a qualitative technique.

Following this, another objective was to explore whether adult and foetal tenocytes displayed discernible differences in their morphology and the influence of particular substrate properties on their behaviour using standard culture media containing serum and serum free culture media. To examine these differences between the tenocytes, cells were seeded onto glass coverslips for 6 hours and 24 hours under three conditions an untreated

control, a phosphate buffered saline treatment and foetal bovine serum treatment and actin cytoskeleton analysed for age-related morphologies. Additionally, the cells were assessed for their cell adhesion strength in response to fluid shear stress. In contrast to the adult tenocytes, which overall showed themselves to be more sensitive to alterations in their environment; the foetal tenocytes remained remarkably unchanged in morphology despite changes to substrate wettability and surface chemistry. Furthermore, foetal tenocytes showed a higher rate of cell attachment across varying shear stress. These findings illustrate that foetal tenocyte morphology and adhesion strength are independent of environmental changes and as such could be exploited for their use in cell therapy applications for regenerative healing of adult tendon injuries.

Finally, tenocytes from mature P1 (post-natal day 1) mice and foetal mice at E15.5 were statically cultured within the lumen of PLGA fibres and placed in a HFB system for 24 hours, 3 days or 7 day time course experiments. To evaluate the tenocyte behaviour after each culture period, a PicoGreen assay to measure the DNA (deoxyribonucleic acid) profile of the cells was conducted, alongside metabolic assays for glucose consumption and lactate production. The results from these assays determined that both adult and foetal tenocytes continue to prefer growing on tissue culture plastic surfaces in contrast to the PLGA fibres in the HFB setup. This shows that improvements still need to be made if tenocytes are to be utilised for cell expansion applications *in vitro* using a polymer biomaterial such as PLGA and a bioreactor culturing system.

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Abbreviations

ANOVA Analysis of variance

AWERB Animal welfare and ethical review body

BM Basement membrane

CAMs Cell adhesion molecules

DAPI 4'6-Diamidino-2-phenylindole dihydrochloride

DMEM Dulbecco's Modified Eagle Medium

DNA Deoxyribonucleic acid

E Elastic modulus

ECM Extracellular matrix

EDTA Ethylenediaminetetraacetic acid

ESCs Embryonic stem cells

FBS Foetal bovine serum

FDA Food and drug administration

bFGF Basic fibroblast growth factor

FN Fibronectin

GAG Glycosaminoglycans

GF Growth factor

GTP Glutamate-pyruvate transaminase

HFB Hollow fibre bioreactor

HSPG Heparan sulphate proteoglycan

IMS Industrial methylated spirit

L-LDH L-lactate dehydrogenase

MAPK Mitogen-activated protein kinase

MMP-1 Matrix metalloproteinase-1

MSCs Mesenchymal stem cells

MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD^+ Nicotinamide adenine dinucleotide oxidised

NADH Nicotinamide adenine dinucleotide reduced

NEAA Non-essential amino acids

NMP 1-Methyl-2-pyrrolidone

P/S Penicillin/streptomycin

PBS Phosphate buffered saline

PGA Polyglycolic acid

PLA Polylactic acid

PLGA Polylactic-co-glycolide

PLLA Poly-L-lactic acid

PVP Polyvinylpyrrolidone

RO Reverse osmosis

RTK Receptor tyrosine kinase

RT-PCR Reverse transcriptase-polymerase chain reaction

Scx Scleraxis

SEM Scanning electron microscope

TCP Tissue culture plastic

TGF-β Transforming growth factor β

TIMPS Tissue inhibitors of metalloproteinases

Tmd Tenomodulin

VEGF Vascuar endothelial growth factor

WCA Water contact angle

Chapter 1 – Introduction

1.1 The demand for tendon tissue engineering

Tendons play a critical role in many common physiological processes and daily activities, such as walking and lifting an object. There is evidence to indicate that tendons were of vital evolutionary benefit in giving *Homo sapiens* a bipedal gait (Bramble and Lieberman, 2004); although it remains indistinguishable as to when tendons such as the Achilles tendon, were first modified in length in humans. Unlike the great apes, humans possess many tendons in their lower limbs, which connect to the muscles and act as springs to generate force carefully. Of these, the most important is the Achilles tendon, which joins the heel to the major plantar flexors of the foot.

Injuries to tendons specifically rupture of the Achilles tendons, were first noted in 1575 but such cases were virtually unheard of until the 1950's. However, since then incidences of tendon injuries such as the Achilles tendon have steadily increased among Western countries (Leppilahti and Orava, 1998). Tendon injuries are now commonplace, for example rotator cuff injuries are the most frequent cause of shoulder pain in the UK (Bupa, 2012) and there are over 3000 cases of flexor tendon injuries in the UK each year (Ibrahim et al., 2013).

Before the 1920's treatment of tendon injuries like the Achilles tendon had been non-surgical methods mainly restraining the injury site using straps and braces (Leppilahti and Orava, 1998). Surgical intervention was introduced as the preferred treatment after the 1920's; despite this, non-surgical approaches made a resurgence five decades later. Several comparison studies were published between the 1970's and 1980's, weighting up the differences between the two routes of treatment for tendon injuries. Since then, surgery has been preferred to treat injuries in the younger population and athletes, whilst conservative treatment is generally reserved for non-athletes (Leppilahti and Orava, 1998).

Amongst the complications that can hamper healing of tendon injuries is fibrotic scar formation. Disproportionate scar formation can impede the proper restoration of the gliding mechanism of tendon, due to the disruption of the collagen fibres at the injury site (Lin et al., 2004). As a result of this, a large number of tendon injuries are never fully healed and the former biomechanical properties of the tissue are lost.

Therefore, to overcome this problem, the mechanisms behind the healing process of tendon injuries have been the focus of much attention by both researchers and clinicians. Part of this focus has been looking to the field of tissue engineering for innovative techniques to address the issue of scarred healing in tendon injuries, especially the potential of stem cells to differentiate towards a tendon cell lineage. It is now well established that foetal injuries undergo scarless healing (Chiquet et al., 2003; Frantz et al., 2010; Namba et al., 1998), and thus the repair process is markedly different. However, there is inadequate understanding as to why this disparity between regenerative healing in foetuses and reparative healing in adults exists. This is especially true of tendon development and the interactions that occur between cells and the surrounding extracellular matrix (ECM).

1.2 Outline of the thesis

This thesis is divided into 7 different chapters, including this chapter (Chapter 1), which outlines the thesis and gives a brief background into the research area. Chapter 2 reviews the literature that has been done in the subject and associated areas. This chapter also encompasses the aims and objectives of the project and the motivation for conducting the work. Following this is Chapter 3, which covers the methods and materials used throughout the experiments and the various protocols and techniques involved. Chapter 4 details the development and optimisation of tendon cell isolation; Chapter 5 assesses the generation dependent variation of the cellular characteristics between adult and foetal tenocytes. Finally, Chapter 6 specifics the culture of tendon cells within a PLGA hollow fibre bioreactor, with Chapter 7 highlighting the overall

conclusions of the work as a whole and provides suggestions of future work and the potential impact within the particular area of tendon tissue engineering.

Chapter 2 – Literature Review

2.1 Introduction

This literature review details the biology of tendon tissue including composition and structure (2.2) to function and development of the tissue (2.3). This is followed by the composition of the extracellular matrix (ECM) and cell-matrix interactions (2.4), age related differences in the healing of tendon injuries (2.5) and existing treatments available for tendon injuries are also described (2.6). The current situation of the role of tissue engineering is covered, with the various factors to consider for tendon repair (2.7) and finally the aims and objectives of the project are listed (2.8).

2.2 Composition and structure of tendon

Tendon is classified as dense regular connective tissue, and this particular type of connective tissue is limited to tendons and ligaments. Dense connective tissue is either classified as irregular or regular. Irregular dense connective tissue can be located within the dermis of the skin, composed mainly of fibroblasts and mostly collagenous fibres arranged unsystematically (Eroschenko, 2008).

The typical appearance of healthy functioning tendon has a white translucent colour to it, and 80% of its composition consists of the extracellular matrix (ECM), which surrounds the cellular components that make up the rest of its composition. Most of these cells are tenocytes, a specialist fibroblast cell type found within tendon, other cells include chondrocytes at the sites of insertion and synovial cells of the tendon sheath (Sharma and Maffulli, 2005).

The structure of tendon is commonly defined as ‘hierarchical’ (Fig. 2.1) and centred on collagen (Sharma and Maffulli, 2005; Voleti et al., 2012; Wang, 2006). At the lowest level of its structure is the collagen fibril, made up of collagen molecules and has a folded appearance in an unloaded state. Fibrils are held together by proteoglycans such as decorin and fibromodulin, which form primary bundles of fibres. Proteoglycans are proteins with glycosaminoglycans

(GAGs) attached, they are known to be highly hydrophilic, allowing cell migration and diffusion of water-soluble molecules (Sharma and Maffulli, 2005).

It is between these collagen fibres that tenocytes are found arranged laterally to the long axis of the tendon (Sharma and Maffulli, 2005; Wang, 2006). The length of the fibrils alters with age as they change from being consistently small in the young, to fluctuating between having small and large length fibrils with age (Wang, 2006). The primary bundles of fibres aggregate to form tendon fascicle, which are organised parallel to the long axis of tendon, similar to the arrangement of the tenocytes. The endotendon shields individual fascicles, which collectively form the tendon tissue and are surrounded by a thin tissue layer known as the epitendon (Voleti et al., 2012), this serves the tissue with its blood, lymphatic and nerve supply (Kastelic Galeski, A., Baer, E., 1978). Vascularisation of tendons is poor (Docheva et al., 2005) and blood flow to the tendons continues to fall with age and higher mechanical loads (Sharma and Maffulli, 2005).

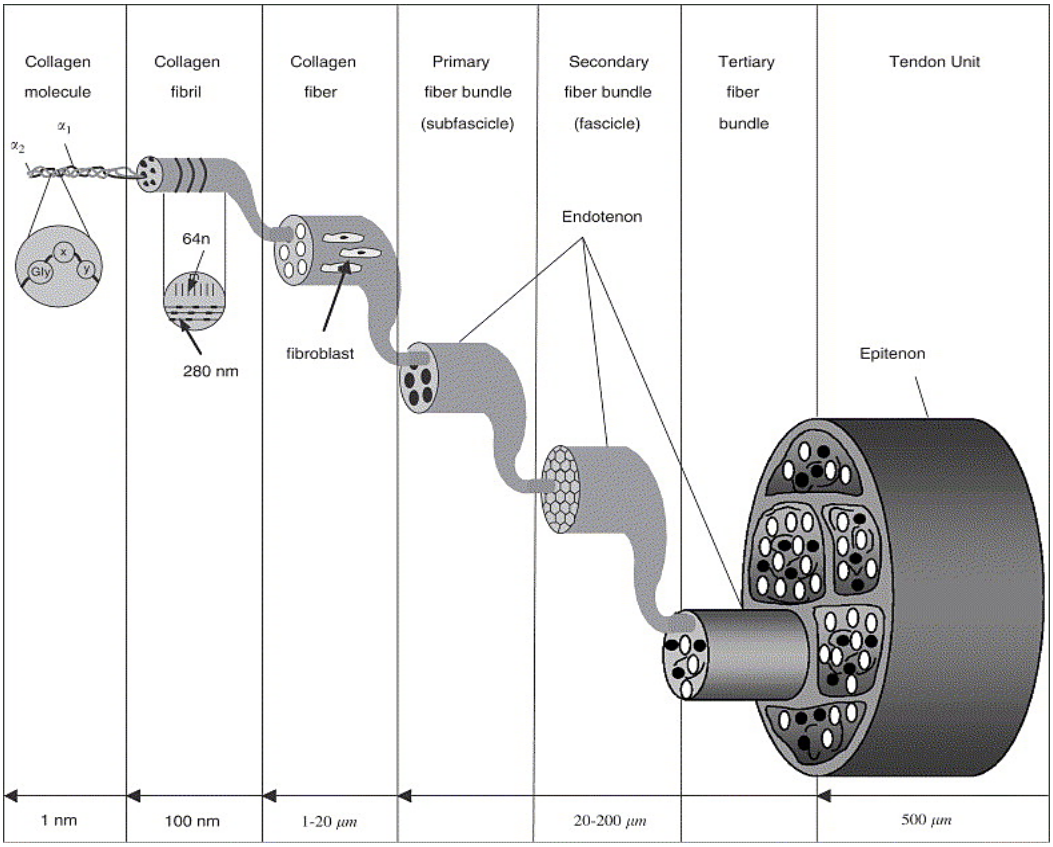


Figure 2.1 Schematic of the hierarchical structure of tendon (Wang, 2006)

Collagen type I is the principal form of collagen that exists in tendon, making up to 80% of its dry mass (Sharma and Maffulli, 2005). Collagen type III is also found in smaller quantities within the ECM usually confined to the endotendon and epitendon. Larger amounts of collagen type III have been identified within aged tendon and tendons that withstand higher levels of stress (Wang, 2006). Research has demonstrated that collagen type III has 'smaller, less organized fibrils' (Lapiere et al., 1977) which could cause tendons to have lower mechanical properties.

The non-cellular component of the ECM is made up of many different constituents including proteoglycans, such as aggrecan and decorin. Aggrecan retains water within the fibrocartilage and decorin can be found on the exterior of collagen fibrils (Wang, 2006). Glycoproteins, (a set of conjugated proteins covalently bonded to one or multiple carbohydrate groups) are further components of tendon ECM, including fibronectin, which like decorin is situated on the exterior of collagens and is known to play a role in the repair process of tendon following an injury (Sharma and Maffulli, 2005). Tenascin-C is a second example of a glycoprotein located in the matrix that together with collagen fibrils provides mechanical support to the ECM (Wang, 2006). Each of the constituents of tendon discussed, both cellular and non-cellular have their role to play in the overall function of tendon, and allow for tendon tissue to be able to withstand numerous stresses.

2.3 Function and development of tendon

Tendons connect muscle to bone at various places in the body, such as the rotator cuff tendon in the shoulders and the Achilles tendon in the heels. Depending on the location of tendon within the body, they will experience a variety of mechanical stresses. Their primary role is to transfer the energy created by muscle contraction to the underlying skeleton resulting in movement of the joints (Lui et al., 2011; Voleti et al., 2012).

2.3.1 Mechanical properties of tendon

As part of this role, tendon is capable of undergoing 'mechanical adaptation'. These adaptations in composition and structure transpire at the cellular level, with tenocytes amending the release of various matrix proteins (Wang, 2006). This behaviour has been observed in cells that were seeded onto relaxed and stressed gel substrates. On relaxed substrates, the tenocytes displayed lower collagen type I expression and increased levels of MMP-1 (matrix metalloproteinase-1), which is involved in matrix degradation. In contrast with this, tenocytes on stressed gels exhibited higher expression of tenascin-C and collagen type XII (Wang, 2006). This highlights that cells have the capacity to react towards the elasticity of a substrate, with cell-matrix interactions being key to conserving the overall tissue structure and function. The importance of cell-matrix interactions will be explored in further detail in section 2.4.6.

The ordered structure of tendon described above, provides tendon tissue with tensile strength (Wang, 2006). At low strain rates, tendon is poor at transferring loads, however this trend is reversed at higher strain rates as the tissue becomes less elastic and more stiff, this behaviour is described as viscoelasticity (Wang, 2006). Relaxed tendon is described as possessing collagen fibrils with a 'crimped or wavelike structure' (Fig. 2.2) (Rees et al., 2006). This crimp structure is reinstated following stretching of tendon via the presence of elastin fibres, although they only constitute approximately 2% of the dry weight of tendon (Wang, 2006). The crimp structure can ultimately influence mechanical properties, as collagen fibres with a large crimp angle are greater at resisting failure than those with a smaller crimp angle (Wang, 2006).

Collagens within the ECM are cross-linked resulting in increased elastic modulus (E) of the tissue and 'reduces its strain at failure' (Wang, 2006). Proteoglycans also have smaller roles to play in contributing to the mechanical properties of tendon. Aggrecan opposes compression and decorin permits 'slippage' (Wang, 2006) of the collagen fibrils experiencing mechanical deformation.

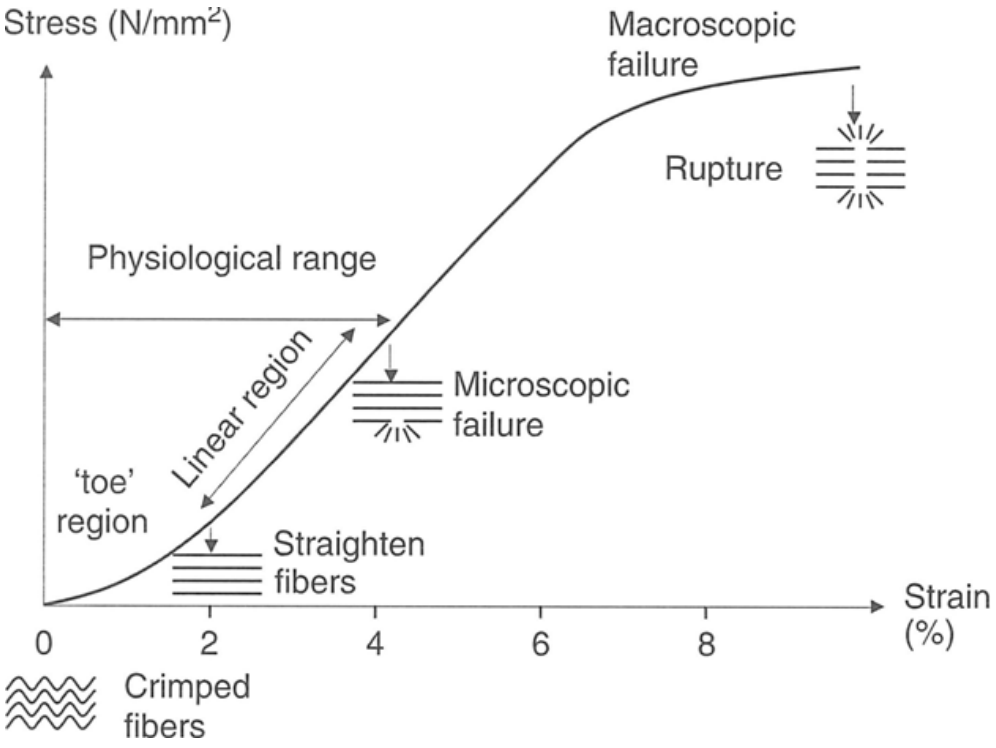


Figure 2.2 Stress-strain curve of tendon tissue fibres (Wang, 2006).

Previous research indicated that beyond 4% strain, tearing of the tendon fibres would occur at a microscopic scale, between 8-10% strain, macroscopic tearing of the fibres takes place (Fig 2.2). However, testing of tendons in animal models has contradicted this, as avian flexor tendons were elastically stretched at 14% strain before tearing occurred (Wang, 2006). Mechanical properties including ultimate tensile strength and Young's modulus of tendon have been shown to decrease with age, and damage to tendons can occur through overloading, which inevitably leads to injury of the tissue. Despite this, it is clear that tendons are well equipped to carry out the function of transferring stresses from muscle to bone.

2.3.2 Embryonic development of tendon tissue

Studies in mice have reported in vitro, tendon progenitor cells begin to express scleraxis (Scx) a transcription factor that is associated with tendon tissue from a early stage at embryonic day 9.5 (E9.5) and onwards (Brown et al., 2014). By E14, the tissue is exposed by muscle activity, indicating that mechanical stimulations are a factor towards tendon differentiation. This is supported by further evidence showing that mesenchymal stem cells (MSCs) that undergo

mechanical stimulus results in the cells expressing tendon cell transcription factors such as scleraxis and TGF-β2, which has been demonstrated as being vital to the initial stages of tendon development. Mechanical signals have therefore, been recongised as having a substantial influence on tendon as it matures in the embryo (Glass et al., 2014). This is further supported by studies that have illustrated that deliberate muscle paralysis leads to deterioration of tendon and debilitating deformation of the muscles and related tissues (Glass et al., 2014).

Other tenogenic cell markers include tenomodulin, a transmembrane protein which presence is detected from E13.5 onwards into adulthood. Therefore the presence of this protein is considered a late marker of tendon tissue formation, Scx positively controls the expression of this protein (Jelinsky et al., 2010).

Prior to the breakthrough of uncovering Scx as a tendon cell marker, the lack of tendon specific markers made the investigation of embryonic tendon development difficult to track (Liu et al., 2011; Yang et al., 2013). Fibroblast growth factors (FGFs) have also be recognised as playing a part in tendon development, especially FGF4 that may have a role in the differentiation process of tendon progenitor cells towards tendon cells. However, it is not considered the trigger for Scx expression in the early stages of the process (Liu et al., 2011). A further tendon specific marker is a glycoprotein Tenascin C, which is expressed upon mechanical loading, where it is found in the highest concentrations, and was the main cell marker used to identify tendon, prior to the discovery of Scx (Yang et al., 2013). Taken together, it is evident that embryonic development of tendon to maturity is informed by an amalgamation of both mechanical cues from the surrounding muscles and chemical cues from the transcription factors, which are expressed; in order to lead the differentiation of the tendon progenitor cells towards mature tenocytes.

2.4 Composition and structure of the ECM

Proteins form a fundamental component of the ECM; these proteins are classified as either structural proteins. Within the ECM, the structural proteins

are elastin and collagens. The non-structural proteins are fibronectin, laminin and tenascin. Other matrix constituents include integrins, growth factors and a group of MMPs (Figure 2.3). Integrins are the vectors for communication between cells and the ECM; the significance of their function is detailed separately in section 2.4.6.

2.4.1 Proteins

Collagens

Presently, there are almost 30 types of collagen that have been discovered, although not all are matrix proteins. As a consequence, collagen is commonly referred to as the most abundant protein within the body, and is found concentrated in the matrix of connective tissues such as tendon and skin. In these tissues, collagen is organised as fibrils, thereby transmitting essential structural integrity for these tissues. In particular, collagen fibrils are produced by collagen types I, II, III, V and XI (Kular et al., 2014).

Collagen type I is the most profuse form, common to almost every tissue type, principally in tendon and skin. The other forms of collagen are distinctive to particular tissues, for example collagen type II is found in cartilage and the cornea, while collagen type III can be found within the walls of blood vessels (Bosman and Stamenkovic, 2003). Elastin is the other structural protein, with its function supportive of collagen; it provides skin and blood vessel walls (Eckes et al., 2010) with the ability to recover from continuous stretching (Frantz et al., 2010).

Fibronectin

Fibronectin (FN) is another protein that has been investigated by researchers, although not as extensively as collagen. It is a glycoprotein with a fibrillar architecture, set within the basement membrane of the ECM, and it has been defined as having a major part to play in both cell adhesion when it is expressed by fibroblast cells (Tanzer, 2006) and the repair of injuries where it arrives in the form of plasma transported within the blood (Eckes et al., 2010). The presence

of FN is also essential to the early stages of embryonic development, (Mao and Schwarzbauer, 2005).

Laminins

Laminin is glycoprotein situated in the basement membrane, originally acknowledged as a component of the ECM of murine EHS sarcoma (Matrigel) (Bosman and Stamenkovic, 2003; Tanzer, 2006). Various tissue types including both muscle and epithelial cells release it. Laminins have an underpinning role in a number of routine cell processes such as adhesion, migration, differentiation and proliferation (Eckes et al., 2010). Furthermore, laminin has been identified as being manifest in certain diseases such as cancer (Bosman and Stamenkovic, 2003). Laminins convey signals via their integrins, mediating between cells and the basement membrane where they are located (Bosman and Stamenkovic, 2003).

Tenascins

Tenascins are a set of ECM proteins, common to connective tissues in locations that endure load. As a result of this, mechanical stimulation has been demonstrated as influencing tenascin expression (Chiquet et al., 2003). Due to its specific expression within load bearing connective tissue, tenascin-C is used as one of the markers to identify tenogenic differentiation of stem cells (Yin et al., 2010). In concurrence to this, a lack of tenascin-C inhibits regeneration processes, as it is positively expressed in the interim matrix following wound damage (Eckes et al., 2010), it is not found to be expressed. Disease is also known to adversely affect its expression (Chiquet et al., 2003), for example it is present during tumour proliferation.

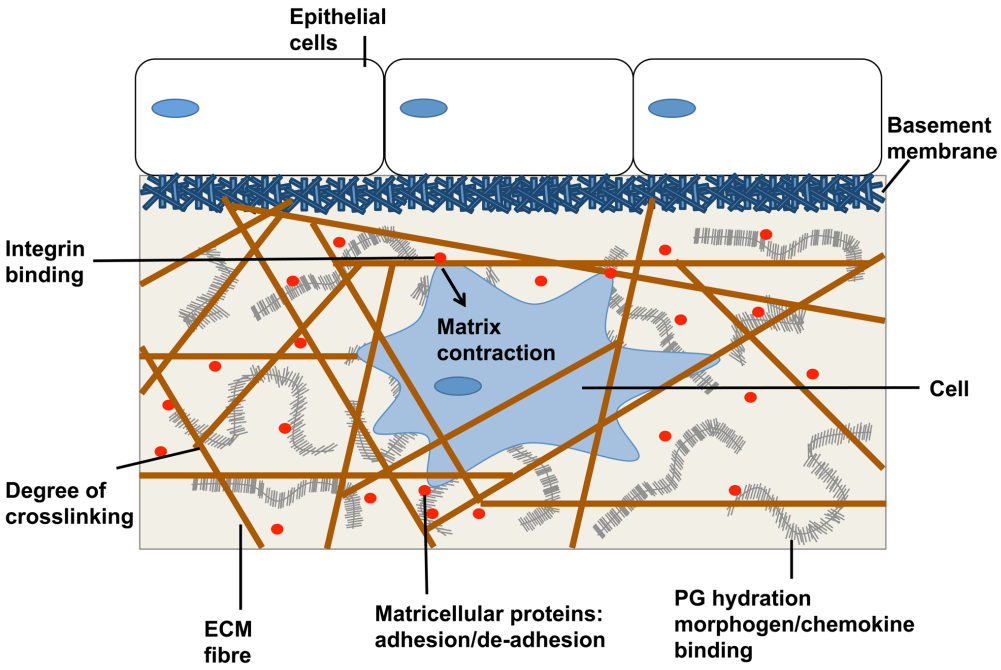


Figure 2.3 Composition and structure of the ECM (Kular et al., 2014)

2.4.2 Growth factors

The ECM is considered to have a wealth of growth factors (GFs) (Eckes et al., 2010; Kim et al., 2011). The involvement of GF's in cell pathways is influenced by various ECM components. Vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and transforming growth factor β (TGF-β) are examples of GFs linked to the ECM (Hynes, 2009). Growth factors can be activated through numerous routes (Kim et al., 2011) including wound healing and tissue remodelling (Eckes et al., 2010). Growth factors themselves can also trigger expression of ECM proteins, an example of this is tenascin-C (Chiquet et al., 2003). Previous investigations of GFs in wound healing have recognised the importance of them in the repair of tendon injuries in adults (Lui et al., 2011; Yin et al., 2010).

2.4.3 Matrix metalloproteinases (MMPs)

Overall in excess of 20 matrix metalloproteinases (MMPs) have been recognised (Bosman and Stamenkovic, 2003; Stamenkovic, 2003); they are the group of enzymes largely accountable for breaking down the ECM. Collectively, MMPs have the ability to degrade each ECM protein, as they are not substrate specific (Bosman and Stamenkovic, 2003). MMPs therefore make a significant

contribution to the constant remodelling that the ECM undergoes. MMP expression is escalated in response to cytokine and growth factor activity, as a result of injury or tissue modification. Due to the powerful action of MMPs they are strictly controlled via three mechanisms; primarily by governing them during transcription, secondly by keeping them dormant when they are not activated, and lastly having tissue inhibitors of metalloproteinases (TIMPs) to prevent unnecessary damage to the matrix (Kim et al., 2011).

2.4.4 Basement membrane

Many ECM proteins can be found in the basement membrane such as collagen type IV, laminins and fibronectin. The FN imparts some tensile strength to the tissue (Kular et al., 2014). The basement membrane is located in blood vessels, epithelial and endothelial tissue, forming a highly organised matrix, with the epithelium heavily reliant on the BM for its function (Kim et al., 2011). As mentioned in previous section relating to laminins, integrins relay communications between the basement membrane and the cells above in relation to cell behaviour (Tanzer, 2006).

2.4.5 Interstitial matrix

Interstitial matrix appears together with the basement membrane; moreover it also is found between connective tissue cells. It's main components include the proteins described above such collagens, elastin and fibronectin (Mitchell et al., 2007). Although in comparison to collagen, it is relatively low in abundance as an ECM protein, FN in fact has the greatest influence over the arrangement of the ECM structure (Frantz et al., 2010).

2.4.6 Cell-matrix interactions

The ECM is a constantly changing environment, with alterations in composition and structure. These modifications arise to adapt to the behaviour of cells, this highlights appreciating the importance of the relationship between cells and the ECM and how they evolve in accordance with each other (Kular et al., 2014). With connective tissue such as tendon, the cells respond to mechanical strain on ECM through cell-matrix adhesions and act accordingly by down-regulating

or up-regulating ECM production. Focal adhesions are the principle channel of communication between the ECM and cytoplasm, which also provide 'strong cell-substrate adhesion' (Chiquet et al., 2003).

Integrins

Integirns are linked to ECM proteins like FN, which then result in binding of cells to the ECM. Integrins are classified as 'transmembrane heterodimers' (Kurtz and Oh, 2012), impacting upon a variety of cell signalling pathways for cell propagation and cell motility, via their connection to actin of the cytoskeleton linker proteins associated with integrin cytoplasmic tails (Bosman and Stamenkovic, 2003; Eckes et al., 2010). Connections have been made between the responses of integrins to the activity of cells in relation to mechanical stimulus (Chiquet-Ehrismann and Tucker, 2011). This is due to the initiation of signalling routes via various mechanisms (Eckes et al., 2010). Integrins form part of a network of other matrix components that are involved with the 'transduction of mechanical forces' including G proteins, receptor tyrosine kinases (RTKs) and mitogen-activated protein kinases (MAPKs) (Wang, 2006).

Cytoskeleton

Similar to integrins, the cell cytoskeleton adapts to the mechanical stimulus imparted by cells upon the ECM. The composition of the cytoskeleton consists of actin microfilaments and microtubules (Kular et al., 2014). Overall, cell-matrix interactions are now fully recognised as underpinning to the mechanotransduction of cells. These interactions contribute to regular cell processes such as development through to abnormal cell behaviour including the formation of cancerous tumours (Bosman and Stamenkovic, 2003).

2.5 Age related differences in the healing of tendon injuries

Per annum there are 30 million tendon and ligament injuries globally (Lui et al., 2011). Many of these injuries are sports related injuries in the young and middle-aged adult populations. For example Achilles tendinopathy is thought to affect half of all athletes, especially runners. Achilles rupture affects between 6-18 people per 100,000 of the UK population (NICE, 2010). However, all sections

of the population are affected by tendon and ligament injuries for example, tennis elbow occurs in 1-3% of the UK population (NICE, 2012). These injuries also occur at a similar rate in senior citizens, and the UK government recently estimated that the number of elderly people will reach almost 19 million by 2050 (Cracknell, 2010). It is evident that such injuries will continue to put pressure on the healthcare service for the foreseeable future.

2.5.1 Classification of tendon injuries

Tendon injuries are classified as acute (tendon rupture), which usually occurs due to trauma or chronic (tendinopathy) resulting from overuse or deterioration with age (Lui et al., 2011; Sharma and Maffulli, 2006). Common to all tendon injuries, the regular structure is compromised and the normal function of the tissue is hard to restore. It was thought that inflammation was a symptom of chronic tendon injuries, but this has now been disputed (Rees et al., 2006) and histology samples demonstrate 'increased cellularity' and disordered collagen fibrils (Voleti et al., 2012) but little or no sign of inflamed tissue (Rees et al., 2006).

This process can take place over a period of time, as tendinopathy is associated with aging. However, tendon rupture could happen randomly, caused by direct trauma or by intense loading. Although evidence from histological samples shows that prior to rupture, alterations in the tissue are similar to those found in cases of tendinopathy. This would indicate that acute tendon injuries are linked with 'degeneration' (Rees et al., 2006; Voleti et al., 2012).

2.5.2 Inflammation stage

The healing process of tendon injuries can be divided into three distinct stages that intersect, starting with inflammation, followed by the proliferative stage, which takes place 48 hours after injury, and ending with the remodelling stage. Inflammation is characterised by the arrival of white blood cells such as erythrocytes and neutrophils, macrophages and monocytes also appear to ingest the necrotic tissue. Tenocytes are encouraged to the injury site to begin

proliferating and collagen type III production begins (Sharma and Maffulli, 2005; Voleti et al., 2012).

2.5.3 Proliferative stage

The proliferative stage begins a few days later and is driven by macrophages and tenocytes. Additionally, it is dominated by large amounts of synthesis; of collagen type III in particular (Sharma and Maffulli, 2005; Voleti et al., 2012), which is formed by tenocytes. This production of collagen type III continues for several weeks. The collagen type III forms the main component of 'a temporary, mechanically inferior matrix' (Voleti et al., 2012), and macrophages are responsible for releasing 'growth factors and direct cell recruitment' (Voleti et al., 2012). Similarly, the amount of water and glycosaminoglycans remains high throughout this stage of healing. The activity that occurs during this phase takes place within a hypoxic setting, and despite the fact collagen assembly is reduced in low levels of oxygen, collagen deposition benefits from higher amounts of lactate (Voleti et al., 2012).

2.5.4 Remodelling stage

The final remodelling stage is instigated after a few weeks but can continue for up to a year after the original injury. It can be further split into a consolidation stage and a maturation stage (Sharma and Maffulli, 2005). The consolidation stage occurs over a period of several weeks, the repair tissue transforms from a cellular composition to a fibrous composition, tenocyte activity continues steadily and the cells and collagen fibrils orientate themselves in parallel with the 'direction of stress'.

Collagen type I is heavily produced in the remodelling process. The maturation stage of the remodelling is defined by the slow adjustment of the fibrous tissue to scar tissue with a decrease in tenocyte activity (Sharma and Maffulli, 2005). This scar tissue, with poor biomechanical properties, is what finally replaces the original healthy tendon tissue. It prevents restoration of the usual gliding mechanism due to adhesion formation, which sees the surrounding tissue merge with the injury site (Voleti et al., 2012).

2.5.5 Scarless healing vs. scarred healing

Present knowledge on the mechanisms of tendon injury and healing, has largely stemmed from both *in vivo* studies of animal models and *in vitro* studies with cell cultures. From animal models, such as sheep (Beredjiklian et al., 2003), researchers have sufficient evidence that foetal tendon injuries undergo scarless healing (Fig. 2.4) and repair at a faster rate than tendon injuries in adults. This scarless healing process has also been observed in other tissue types including skin, bone and cartilage (Chiquet et al., 2003; Frantz et al., 2010; Namba et al., 1998).

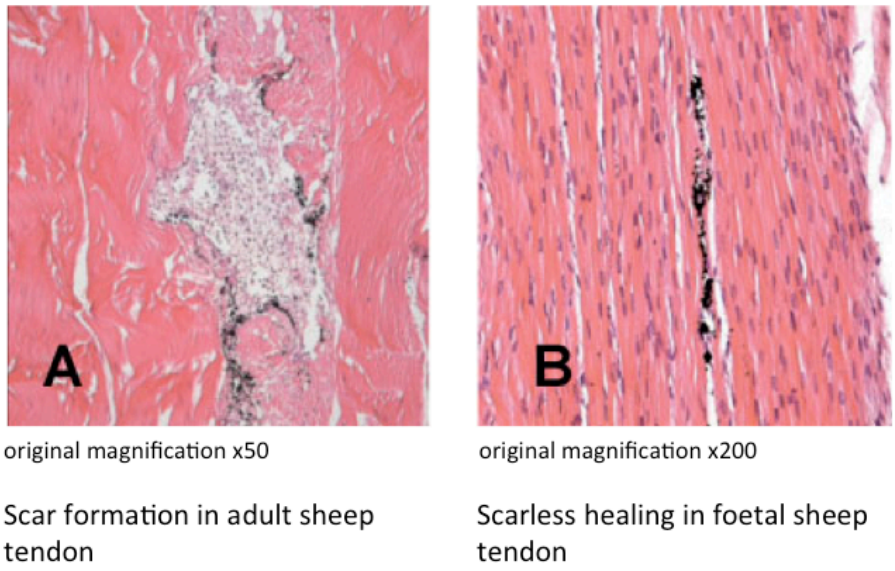


Figure 2.4 Comparison of adult and foetal tendon healing in sheep (Favata et al., 2006)

The healed tendon injuries of foetuses were restored 'without gross or histological abnormality' (Voleti et al., 2012). Whilst the healed tendons of the adult sheep recovered inadequately, displaying random collagen fibres, disjointed fibre bundles and substantial amounts of granulation tissue. Further investigations have shown that the ability of foetal tendon to heal in a scarless manner is independent of the intrauterine environment (Favata et al., 2006). Therefore this implies that the scarless healing process can be attributed to the properties of the tissue itself and the relationship between the cells and surrounding extracellular matrix.

Progressively with age, there are alterations that occur both biologically and mechanically resulting in the healing properties of the tissue becoming steadily worse (Voleti et al., 2012). Collagen synthesis decreases, and as a consequence the thickness of the collagen fibrils becomes inconsistent, the ability of the fibrils to resist failure is also reduced as their crimp angle is lowered (Voleti et al., 2012). Overall aged tendon is at a higher risk of tearing, due to diminished mechanical properties as the tissue becomes stiffer. However, there have also been challenges to the concept that mechanical properties of healing tendon tissue declines with age (Dressler et al., 2006).

Existing research has mainly focused on comparing the biochemical and biomechanical age-related differences between young and aged samples. Research at the cell-matrix level has not concentrated at examining the differences from foetal and adult sources. Therefore, mechanisms behind the regenerative healing capability of foetal tendon against reparative healing of adult tendon remain poorly understood.

2.6 Treatments for tendon injuries

Repair of tendon injuries either involve surgical intervention or conservative techniques. Conservative treatments are usually the first option; examples of these include corticosteroid injections, anti-inflammatory drugs and low intensity ultrasound (Rees et al., 2006). The main objective of such methods is pain management and they are often lengthy processes, with little to no consideration given to restoring the biochemical or biomechanical functions of the tissue. When these treatments are regarded as unsuccessful, surgery is the only viable alternative.

2.6.1 Conservative treatment

Researchers have questioned the effectiveness of anti-inflammatory drugs, as there is little to no evidence to support how useful they are in restoring tendon tissue (Wang and Shapiro, 1997). Administration of steroid injections is commonplace, however prior studies indicate they only provide interim pain relief for both rotator cuff injuries and cases of tennis elbow (Rees et al., 2006).

Additionally, there have been reports of problems following steroid injections, for example, tendon rupture (Kleinman and Gross, 1983) and a decrease in tendon strength in certain animal models (Kapetanos, 1982). Overall, the value of such conservative treatments is inconclusive, largely due to the lack of robust clinical studies (Speed, 2001) backing their use.

2.6.2 Surgery

With surgery there are two possible routes to repair the injury site; the first is through sutures, which are used to seal the injury site. This is commonly used to treat damage in flexor tendons and rotator cuff tendons (Voleti et al., 2012). The second route is grafting of tendon tissue, using the patient's own tissue (autograft) or via another donor (allograft). Although, it is preferable to use an autograft as there are fewer problems such as an immunological reaction; there are obvious drawbacks to such surgical methods, including that the replaced tendon is different to the original tissue, and there is limited availability of such graft tissue for both autografts and allografts (Yin et al., 2010).

2.6.3 Mechanical stimulation

It is worth noting that several authors have explored the benefits of implementing mechanical stress to healing tendon injuries. Encouraging 'mechanical stimulation' of the tendons post inflammatory phase has been demonstrated as having numerous positive effects on the tissue, from 'increasing tensile strength', to repairing the gliding mechanism and rebuilding the morphological properties of tendons (Voleti et al., 2012). Measured mechanical stimulation of the healing tendon is thought to prompt accelerated repair via tenocyte propagation, collagen synthesis, further release of additional ECM proteins and the action of MMPs in governing the turnover of the extracellular environment (Voleti et al., 2012). Furthermore, this highlights the significance of mechanotransduction in influencing the relationship between tenocytes and the surrounding matrix at the cellular level (Voleti et al., 2012; Wang, 2006).

Conventionally, clinicians have focused on pain management, and a successful treatment solution for tendon healing that restores the original biomechanical and biochemical properties of the tissue has yet to be found. All this data highlights the demand for developing better solutions for treating these injuries. Part of this, involves gaining a greater understanding behind the age related differences in the healing process of tendon injuries, and how tissue engineering can play a role in this. As a consequence, tissue engineering is being actively pursued as a possible future treatment option.

2.7 Role of tissue engineering for tendon repair

Regenerative medicine is increasingly becoming a potential therapy to replace original tissue, with tissue engineering providing the method to produce prospective constructs for this therapy. The concept involves implantation of cells within a scaffold construct along with the required biomolecules. Presently, there are a few examples of such applications in a clinical setting, including artificial skin (Yin et al., 2010). The necessary components for tendon tissue engineering, demand specific sourcing of cells, scaffold material, and expansion technique. Tendon is an appealing tissue candidate for tissue engineering applications, namely due to its avascular nature, which is an issue for other tissue types.

2.7.1 Cells

One of the most important factors to consider for tendon tissue engineering is the type of cells to be used. This means considering their availability, 'proliferation potential' (Yin et al., 2010), how they create the ECM, their cell-cell interactions and their cell-matrix interactions. Of the cells that have been used, they can be classified as fibroblasts (tenocytes and dermal fibroblasts), MSCs and embryonic stem cells (ESCs).

Tenocytes

The problems associated with using tenocytes as the cell source has been well reported within the literature (Lui et al., 2011). The main complications are firstly that the principal constituent of tendons is their extracellular matrix; as a

result the overall cell population is relatively low. This has a detrimental impact on acquiring a sufficient number of cells for seeding onto scaffolds for implantation to be deemed successful (Lui et al., 2011).

Additionally, they are known to experience phenotypic drift so they are limited in the number of passages they can be used (Almarza et al., 2008). This can be observed by measuring the expression tendon specific genes such as tenomodulin (Tmd). This is a transmembrane glycoprotein that is essential for tenocyte propagation and development of tendon (Docheva et al., 2005). Ultimately, as detailed in 2.3.5, the healing ability of adult tenocytes is compromised (Lui et al., 2011); therefore other cell types are preferable for use in tissue engineering scaffolds.

Stem cells

The most popular two cell sources that are being explored for tissue engineering applications are embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs). The distinction between the two types of stem cells is that MSCs have a pluripotent capacity and can differentiate into different mesenchymal tissue including tendon (Voleti et al., 2012). ESCs have a greater potency facility to develop into all tissue types within the body (Lui et al., 2011).

MSCs can be sequestered from numerous sites within the body such as bone marrow (Yin et al., 2010). Due to their availability, MSCs have been widely explored as an option in the repair of tendon/ligament injuries, and there have been reports of varying success (Awad et al., 2003; Kryger et al., 2007) of MSCs differentiating into tenocytes following their implantation into tendon injury sites via scaffolds such as collagen gels. However, MSCs also have a tendency to differentiate into bone, which is undesirable for tendon regeneration. This is an example of an issue that needs resolving; other parameters also require refining including improving the mechanism of differentiation MSCs towards a tenogenic phenotype (Lui et al., 2011; Yin et al., 2010).

ESCs may be more preferable as a cell source for tendon repair; there is already evidence to demonstrate that the regeneration of tendon within the foetus is

intrinsic to the cells and independent of the surrounding foetal environment. Thus ESCs may be the ideal cell type to fully restore injured tendons. Yet, there is little information on successful differentiation of ESCs into tenocytes.

This is partially due to the absence of definitive tenocyte cell markers; there are a select few genetic markers that are identified with tendon cells. Furthermore they are also expressed in other tissue types. Scleraxis (Scx) is widely accepted as an appropriate genetic marker for tendon, despite the fact it can also be detected in ligament tissue (Hoffmann et al., 2006). Other common markers used for tenogenic differentiation of stem cells are collagen types I and III, due to their high abundance within the ECM of tendon, tenomodulin and tenascin-C (Morita et al., 2012). Additionally, with ESCs there is a possibility of 'teratoma formation' i.e. tumour development (Lui et al., 2011).

Controlling the differentiation profile of stem cells towards tenocytes is key to developing tissue engineering applications for tendon regeneration. There are several ways that this has been investigated. Namely employing co-culture methods, manipulating scaffold materials and exploring the influence of biological elements such as growth factors (Yin et al., 2010).

2.7.2 Scaffold material

There are a number of factors to consider when selecting a scaffold material for tissue engineering applications; these properties include biocompatibility to the surrounding tissue, mechanical properties in particular the stiffness, the influence on cell behaviour to encourage attachment, proliferation and differentiation (in the case of stem cells) (Jafari et al., 2015) and the degradation rate of the material over time (Rowland et al., 2012). Biomaterials scaffolds predominately fall within two categories. These are synthetic and biologically derived materials, including ECM derived scaffolds. Previous studies have investigated using various materials for repair of tendon and ligament injuries.

Synthetic scaffolds

Typically synthetic scaffolds are polymers polylactic acid (PLA), polyglycolic acid (PGA) and their co-polymers such as polylatic-co-glycolide (PLGA). This literature

review will focus on the co-polymer PLGA, as it was the scaffold material used within this project. However, there are other synthetic polymer scaffolds that have been employed for tissue engineering applications including but not limited to poly-L-lactic acid (PLLA) and poly-ε-caprolactone (PCL) (Lomas et al., 2014; Zhang et al., 2012).

The characteristics of these two polymers differ, with PGA breaking down faster but possessing hydrophilic qualities. In contrast, PLA is considered hydrophobic and a lengthier degradation rate. The co-polymer, PLGA has the advantage of allowing for greater control over the degradation rate by altering the ratio of the monomers. Furthermore it has demonstrated biocompatibility and approval for use within the body along with PLA and PGA (Gentile et al., 2014).

Improvements to enhance the design and properties of PLGA have been investigated. An example of this is to modify the surface of the scaffold fibres with adsorption of growth factors such as basic fibroblast growth factor (bFGF) (Zhang et al., 2012) and fibronectin (Bassetto et al., 2011). Basic fibroblast growth factor is known to promote expansion and differentiation of stem cells towards tendon cells resulting in an increase in the presence of ECM tendon proteins and formation of collagen from the cells; while fibronectin acknowledged as stimulating cell adhesion and proliferation as an ECM protein (Lomas et al., 2014).

A further example of how the design of scaffolds has been explored is in relation to the fibre diameter, with various groups trialling the effects of how altering the size of the fibre influences cell behaviour (Jafari et al., 2015), in particular cell phenotype and ECM formation (Bashur et al., 2006). The size of fibres used for scaffolds is dictated by several parameters, which in turn are dependent on the fabrication technique selected. The diameter of collagen fibres within connective tissue like tendon commonly has a division between a ‘bimodal diameter’ at the nanometre scale ranging from 40-400 nm differing depending on the tissue, individuals and is subject to disruption following scar formation during the healing of an injury (Zhang et al., 2012).

Biological scaffolds

Typical materials used by others are collagen and silk, and ECM derived scaffolds, each of these scaffold types has shown varying rates of success for tissue engineering applications. Collagen is the main constituent of tendon tissue, therefore is viewed as a favourable option as a biological scaffold material. Furthermore, it has several advantages of being compatible for use within the body due to it being a naturally occurring protein and supports cell adhesion and proliferation. Collagen as a scaffold material is often merged with other polymers to heighten its mechanical properties (Bassetto et al., 2011; Ho et al., 2014). This is one of the limitations of the material, along with restrictions on the degree to which it can be processed as a scaffold.

Silk as a scaffold material has been gaining in popularity, it is biodegradable and in contrast to collagen it possesses a natural high mechanical strength. It has been successfully been implemented together with mesenchymal stem cells to repair ligament damage in animal models. Despite this, there are some drawbacks to using silk for tissue engineering applications largely due to the expense and time involved in handling of the raw silk fibres prior to processing which poses a question regarding how viable it is to scale up for clinical use (Tamayol et al., 2013).

Decellularised scaffolds, also referred to as ECM derived scaffolds can offer a potentially desirable solution to repair injured tissue sites. These scaffolds can clearly foster cell proliferation whilst still retaining the natural structural tissue environment (Bassetto et al., 2011). In order for decellularised scaffolds to be effective, it is paramount that all traces of previous cellular content is removed as thoroughly as possible. This is required to prevent any unfavourable immunological reaction being triggered once the scaffold is inserted into the host. However, a balance needs to be maintained against this to ensure that the method used to eliminate the cellular matter is not at the expense of the structural and mechanical integrity of the scaffold. Presently, an ideal decellularisation technique has yet to emerge and investigation into this is still vital (Ho et al., 2014).

2.7.3 Expansion technique

Tissue culture plastic

Commonly cells are proliferated *in vitro* using tissue culture plastic (TCP) in the form of a culture flask that has a rigid surface, in the absence of any mechanical stimulation, an excess of oxygen and a weak cell culture media. These conditions are detrimental for tissue engineering applications that are reliant on an adequate number of initial cells. Furthermore, the environment fostered with tissue culture plastic promotes phenotypic drift and cell senescence (Spanoudes et al., 2014). Therefore to expand a population of cells taken from a patient an alternative viable solution is required, of which some are discussed in the sections below.

Spinner flask bioreactors

Spinner flask bioreactors have been used sparingly as a technique to expand cells for tissue engineering applications. However, there are examples where the method has been tested for both tenocytes and bone cells (Rauh, Juliane Milan, Falk Gunther, Klaus-Peter and Stiehler, 2014; Stich et al., 2014b). These particular bioreactors work by having the cells seeded on scaffolds at the heart of the reactor, connected to a needle within the lid of the flask. The culture medium is then passed through the reactor, which is continuously stirred using a magnetic stirrer at the base of the flask; a level of shear stress is generated which is dependant on the actions of the stirrer (Rauh, Juliane Milan, Falk Gunther, Klaus-Peter and Stiehler, 2014). Although the design and setup of a spinner flask bioreactor is relatively easy and low-cost, there are drawbacks to employing the system to proliferate cells in vitro. These downsides include the possible formation of an impenetrable outer layer of cells, which results in cell senescence in the middle of the scaffold construct to limited oxygen and nutrient supplies (Rauh, Juliane Milan, Falk Gunther, Klaus-Peter and Stiehler, 2014).

Hollow fibre bioreactors

It has been over four decades since a hollow fibre bioreactor (HFB) was first utilised for cell expansion in vitro, therefore this is not a new technology in tissue engineering. The method has been trialled on proliferation of several different cell types such as hepatocytes and lymphocytes (Shipley et al., 2011) however it has not been tried previously for tendon cells. It has also be used in other applications, for example bioartificial organs and for *in vitro* modelling (Wung et al., 2014). The arrangement of a HFB is relatively simple consisting of a glass unit that can house a single fibre or numerous fibres which are formed from a porous biodegradable polymer; with typically the cells either being seeded onto the outer surface of the fibres, inside the lumen of the fibres or suspended within a gel surrounding the fibres (Chapman et al., 2014; Wung et al., 2014).

There are many reasons to why HFBs are seen as a suitable vehicle for cell expansion, the key advantage is the surface area to volume ratio (Shipley et al., 2011; Wung et al., 2014) which means that it is possible to cultivate a higher population of cells *in vitro* compared to conventional cell culture techniques. In addition to this the hollow fibres used have a high degree of permeability, and the arrangement of the fibres in a HFB means that it can facilitate in adequate transfer of nutrients and waste products between cells and the culture media (Whittaker et al., 2009; Wung et al., 2014). Despite the fact that HFBs have been used for many years, little is understood about the effect of different parameters upon the cells such as the initial cell seeding density and shear stress generated by the perfusion of media in or a change of media under static conditions (Chapman et al., 2014).

2.7.3 Additional factors

Co-culture

Co-culture has been validated as stimulating positive differentiation of stem cells towards the desired lineage; it has been shown with co-culture of stem cells with both ligament fibroblasts and tenocytes. Most recently, co-culture of

epithelial cells from amniotic fluid (AECs) with foetal tenocytes or explant tissue resulted in correct differentiation of the AECs to a tenocyte phenotype (Barboni et al., 2012). This particular technique has been proven to work, as it allows for communication between the differing cell populations and could be an effective way of stimulating tenogenic differentiation via molecular signalling, although the exact mechanism is not fully understood (Yin et al., 2010).

Substrate elasticity

There has been much interest in the influence of substrate topography over stem cell differentiation, in particular the stiffness or elasticity of a substrate. This relationship between cells and the underlying matrix is referred to as mechanobiology (Wang, 2006). As mentioned earlier, as part of its role, tendon responds to mechanical load. It does this in a variety of ways at the cellular level, changing its 'gene expression, protein synthesis and phenotype' (Wang, 2006).

2.8 Aims and objectives

The literature review highlights the pressing need to find a solution to a demanding and growing challenge of trying to effectively heal and restore tendon injuries. It is acknowledged that foetal tissue has the ability to undergo scarless, regenerative healing; however there are no clinical cell-scaffold therapies that exist to replace current surgical solutions. Using this knowledge, the aims of this research were as follows:

1. Successfully isolate and culture adult and foetal tenocytes
2. Characterise generational variations between adult and foetal tendon cell morphology
3. Explore culturing tenocytes within a HFB (Hollow Fibre Bioreactor) system using PLGA fibres

With the objective that improving the understanding of the characteristic differences between adult and foetal tenocytes together with designing a successful cell delivery system could ultimately lead to influencing and advancing repair of adult tendon injuries.

Chapter 3 – Materials and Methods

3.1 Introduction

This chapter outlines the specific materials and methods used for experiments conducted in this thesis. Firstly, materials are itemised in relation to particular experiments, followed by the details of experimental methods used, which also outlines the preparation of materials for each experiment. Lastly, the specifics of the analytical methods that were used are described.

3.2 Materials

Below the materials used (listed together with their supplier and catalogue number) are divided into the subsequent sections: cell culture (Table 3.1), cell morphometric analysis (Table 3.2), contact angle measurements (Table 3.3), shear stress assay (Table 3.4) PLGA fibre preparation (Table 3.5) and hollow fibre bioreactor preparation (Table 3.6).

Table 3.1 Materials for cell culture

Material	Supplier	Catalogue number
15ml Falcon tubes	Fisher	11849650
50ml Falcon tubes	Fisher	11819650
35mm Tissue culture dishes	Appleton Woods	BC150
6 well plates	Fisher	10578911
100µm Corning cell strainers	Fisher	15380801
Antibiotic-antimycotic (100X)	Life Technologies	15240-062
Collagenase Type IV	Life Technologies	17104-019
Dispase II	Life Technologies	17105-041
Dissecting scissors (sharp, straight)	Sigma	Z265977

Dulbecco's Modified Eagle Medium (DMEM) – Glutamax	Fisher	11574516
Dulbecco's-PBS (Phosphate buffered saline)	Sigma-Aldrich	D8537-6X500ML
Foetal bovine serum (FBS)	Fisher	10664083
Haemocytometer slides	Una Health	87144
Micro-dissecting forceps (very fine point)	Sigma	F43767
Non essential amino-acids (100X no L-Glut)	Fisher	12084947
Penicillin/streptomycin (P/S)	Fisher	11548876
Scalpel blade No 22 (non-sterile)	Fisher	11792724
Sodium Pyruvate (MEM 100mM)	Fisher	11530396
T25 flasks	Appleton Woods	BC300
Trypan blue solution (0.4%)	Thermo Scientific	15250-061
Trypsin-EDTA (0.05%)	Fisher	11580626

Table 3.2 Materials for cell morphometric analysis

Material	Supplier	Catalogue number
DAPI 4'6-Diamidino-2-phenylindole dihydrochloride, nuclei stain	Sigma-Aldrich	D9542

Dried skimmed milk powder	Morrisons	N/A
Dulbecco's-PBS (Phosphate buffered saline)	Sigma-Aldrich	D8537-6X500ML
Fluorescein Phalloidin	Life Technologies	F432
Formaldehyde	Sigma-Aldrich	F8775
Triton X-100	Sigma-Aldrich	93443
Trypan blue, 0.4%	Life Technologies	15250-061
6 well plates	Fisher	10578911
Glass coverslips (18mm x 18mm)	Scientific Laboratory Supplies	MIC3100

Table 3.3 Materials for contact angle measurements

Material	Supplier	Catalogue number
6 well plates	Fisher	10578911
Dataphysics SNS-D051/025 38.1mm length disposable needle	Lab Unlimited	D-6000121
Dulbecco's-PBS (Phosphate buffered saline)	Sigma-Aldrich	D8537-6X500ML
Foetal bovine serum (FBS)	Fisher	10664083
Glass coverslips (18mm x 18mm)	Scientific Laboratory Supplies	MIC3100

Table 3.4 Materials for shear stress assay

Material	Supplier	Catalogue number
Polypropylene female luer	Cole-Parmer	30800-08
Polypropylene male luer	Cole-Parmer	30800-24
Scalpel blade No 22 (non-sterile)	Fisher	11792724
Waton Masrlow 5m marprene tubing	Watson Marlow	902.0064.J16UOM EA 30378
Watson Marlow transparent tubing	Watson Marlow	913. A032016

Table 3.5 Materials for PLGA fibre preparation

Material	Supplier	Catalogue number
1-Methyl-2-pyrrolidone (NMP)	Acros Organics	127630025
Poly(lactic-co-glycolic acid) (PLGA)	Evonic Industries	99024545

Table 3.6 Materials for hollow fibre bioreactor preparation

Material	Supplier	Catalogue number
Micro-dissecting forceps (fine point)	Sigma	F43767
Peroxide cured silicone tubing I.D. 3.2mm, O.D 6.4mm, 25ft	Cole-Parmer	06411-67
Polypropylene male luer	Cole-Parmer	30800-24
Scalpel blade No 22 (non-sterile)	Fisher	11792724
Silicon glue (Sicoset 151)	ACC Silicones	N/A
Stopcocks with Luer Connections; 1-way; male lock	Cole-Parmer	30600-00

3.3 Experimental methods

3.3.1 Cell source

Tenocytes were isolated from the Achilles tendon tissue of CD-1 adult female mice (10-14 weeks old), their foetuses at embryonic day 15.5 (E15.5) and CD-1 P1 (Post-natal day 1) mice pups that were kindly provided by the laboratory of Dr. Robert Williams in the Department of Biology and Biochemistry. All work with murine tissue followed the University of Bath's required regulations and received prior approval from the Chair of the Animal Welfare and Ethical Review Body (AWERB) at the University of Bath. Only cells between passages 1-4 were used in the experiments. A MG-63 (human osteosarcoma) cell line (Sigma) was sourced from a prior stock kept in liquid nitrogen storage by the Tissue Engineering research group.

3.3.2 Tendon tissue isolation

The Achilles tendons from CD-1 adult female mice (10-14 weeks old), their foetuses (E15.5) and CD-1 P1 mice pups were isolated with the use of a stereo microscope (Leica MZ8) and a horizontal laminar flow workstation (Microflow) and washed in a Petri dish with 1.5ml sterile PBS (phosphate buffered saline solution) with 1% (v/v) antibiotic-antimycotic. The isolated tissue was then finely dissected to approximately 1-2mm pieces using a scalpel blade, dissecting scissors and a micro-dissecting forceps, and transferred to a 15ml Falcon tube in 3ml PBS with 1% antibiotic-antimycotic until cell culture was established.

3.3.3 Media preparation

Both the media for tenocyte culture and MG-63 culture contained 87% DMEM Glutamax supplemented with 10% (v/v) non-heat inactivated foetal bovine serum (FBS), 1% (v/v) non-essential amino acids (NEAA), 1% (v/v) sodium pyruvate and 1% (v/v) penicillin/streptomycin. For the serum-free experiments, FBS was excluded from the media composition, containing 97% DMEM supplemented with 1% (v/v) non-essential amino acids (NEAA), 1% (v/v) sodium pyruvate and 1% (v/v) penicillin/streptomycin.

3.3.4 Cell culture techniques

Primary explant culture

Upon isolation of the tendon tissue individual pieces of adult and foetal tissue were washed again in sterile PBS with 1% (v/v) P/S before being transferred to individual wells of a 6 well tissue culture plate, (also washed in PBS), and adding 3ml culture medium containing 87% DMEM, 10% FCS, 1% NEAA, 1% Sodium Pyruvate and 1% P/S. The cultures were maintained at 37°C in a humidified, 5% CO_2/95% air incubator (Galaxy S, Wolf Laboratories). Outgrowth of cells was then observed periodically, with medium changed every 7 days. All cell cultures were carried out inside a sterile Class II laminar flow hood (Microflow Advanced Biosafety Cabinet, Heraeus HeraSafe Biohood).

Explant culture with glass coverslips

The tendon tissue was initially isolated as previously described above, followed by adding multiple pieces of adult and foetal tendon explant tissue (approximately 1mm x 1mm) to 6 well plates. Prior to the culture medium being added to the wells, in half of each of the 6 well plates, the tissue pieces were allowed to air-dry under the laminar flow hood for 5-10 minutes (Tsai et al., 2012) before 18mmx18mm sterile glass coverslips were placed over the explant pieces (Chard et al., 1987). Then 1.5ml of culture media was added per well and cells were cultured until ready to be passaged, media was changed every 7 days.

Explant culture with scoring

The tendon tissue was initially isolated as previously described, in the other half of each 6 well plate, the bottom of each well was scored by making a number of scratches with a sterile scalpel (Bonifacino et al., 2009). Then multiple pieces of adult and foetal tendon explant tissue (approximately 1mm x 1mm) were added and cultured in separate 6 well plates with 1.5ml culture medium, until reaching confluence and passaged. Media was changed every 7 days.

Enzymatic disaggregation

The tissue was dissected as before and cut into smaller pieces using a sterile scalpel. Adapting from previous protocols (Bi et al., 2007; Zhang and Wang, 2010), the tissue pieces were then either incubated at 37°C for 1 hour, in 2ml of both enzymes consisting of 1ml of diluted dispase (4mg dispase in 1ml PBS) and 1ml of diluted collagenase type IV (3mg collagenase type IV in 1ml PBS). Or similarly, a separate method of digesting the tendon tissue in 2ml of 0.05% trypsin for 1 hour at 37°C was done. For a short period following the exposure of the tendon tissue to the enzymes, the digested tissue solution was passed through a 100µm nylon mesh cell strainer. In both cases, with and without the use of cell strainers, the tissue was then re-suspended in standard culture medium, and centrifuged (ALC Centrifuge PK20) at 3000rpm for 10 minutes.

Following this, the supernatant was removed and the cell pellet re-suspended in fresh medium. The cell suspension was then added to a T25 flask (Corning) along with 5ml of standard culture media and incubated at 37°C in a humidified, 5% CO_2/95% air incubator, with media changed every 7 days. Cell proliferation was observed until they were ready for passaging. Upon reaching confluence the cells were treated with 2ml 0.05% trypsin/EDTA and subcultured.

MG63 cell culture

A cryovial containing MG63 cells was removed from liquid nitrogen storage. The contents were thawed out in a water bath at 37°C for 2 minutes. The cell suspension was then transferred to a 50ml centrifuge tube and 15ml of standard culture medium were added. The cell suspension and medium were added to a T75 flask (Corning) and incubated at 37°C in a 5% CO_2/95% air incubator. Cells were seeded at 20,000 cells/cm^2 in 12ml of standard culture media, which was changed every 7 days. Upon reaching confluence the cells were treated with 5ml 0.05% trypsin/EDTA and subcultured.

3.3.5 Cell morphology assays

Cell culture with serum supplemented media

Prior to cell seeding, 18mm x 18mm glass coverslips (Scientific Laboratory Supplies) were sterilized through autoclaving, after which one coverslip was incubated in phosphate buffered saline (PBS) overnight; another was incubated in foetal bovine serum (FBS) overnight, a third coverslip was left untreated to provide a control. Adult and foetal tenocytes were seeded at a density of 5,000 cells/cm^2 and cultured for two different time points: 6 hours and 24 hours onto the 3 glass coverslips that had undergone different pre-treatments in a 6 well plate with each time point experiment repeated in triplicate.

Cell culture with serum free media

The 6 hour and 24 hour cell morphology assay were repeated as outlined above, with the following alterations in protocol: adult and foetal tenocytes were cultured in T25 flasks at a seeding density of 20,000 cells/cm^2 in 5ml of standard culture media. Before seeding cells onto glass coverslips in 6 well plates, the standard culture media was aspirated off from the flasks and the cells were subjected to 2 short washes with PBS. The PBS was aspirated off and 5ml of serum free media was added to the cells and incubated overnight. At the same time, coverslips were prepared either by coating in 1ml of PBS overnight, 1ml of FBS or left untreated. Subsequently, the cells were trypsinised (serum free media was used throughout) and seeded at a density of 5,000 cells/cm^2 onto individual coverslips within 6 well plates under each of the 3 conditions and incubated in 1.5ml of serum free media, at two time points of 6 hours and 24 hours.

3.3.6 Cell expansion in Hollow Fibre Bioreactor

Polylactide-co-glycolide (PLGA) fibre preparation

The PLGA used was composed from a 75:25 lactide to glycolide ratio, 20% of this was dissolved in 80% 1-methyl-2-pyrrolidinone (NMP) (w/v). PLGA hollow fibres used in experiments were kindly provided by Samuel Acott of the tissue engineering research group; having previously been formed through the

process of spinning, a technique practiced within the research group (Fig. 3.1). The spinning rig and collection point were filled with RO (reverse osmosis) water, pressure was set at 2 bar and the needle was attached to the spinneret. Initial water flow through the spinneret is set at 12ml/min. The flow rate was then reduced to 7-9ml/min, and then the PV valve was partially opened to release the polymer. The polymer solution coagulated on contact with the water flow out of the spinneret and into the water bath of the spinning rig. The spinneret was then lowered into the water bath, so that the needle was below the water surface.

The fibre formed was then guided over the rollers of the rig, into the second water bath and onto the second roller. The pick-up motor was switched on to the lowest setting and the fibre guided under and over the roller into the RO water of the collection point. The quality of the PLGA fibre formed was continually monitored throughout the process by visual inspection, to check for example consistency in fibre diameter and lumen size.

Hollow fibre bioreactor design and set-up

Cell culture was done inside PLGA hollow fibres, which were housed within glass bioreactors. The bioreactors had 2 side ports to allow for injection of media (Fig 3.2), the fibres were held in place via silicon gaskets and silicon glue. The glass bioreactors were autoclaved together with sets of silicon rubber gaskets and silicon tubing before being assembled for experimental use. The external ports of the bioreactors were closed off using silicon tubing and one-way valves, which were sterilised in 70% IMS overnight. The PLGA fibres to be inserted inside the reactors were cut to lengths slightly longer than the reactor, with an extra centimetre on each end. The fibres were lightly sprayed with 70% industrial methylated spirit (IMS) before being inserted inside the reactors.

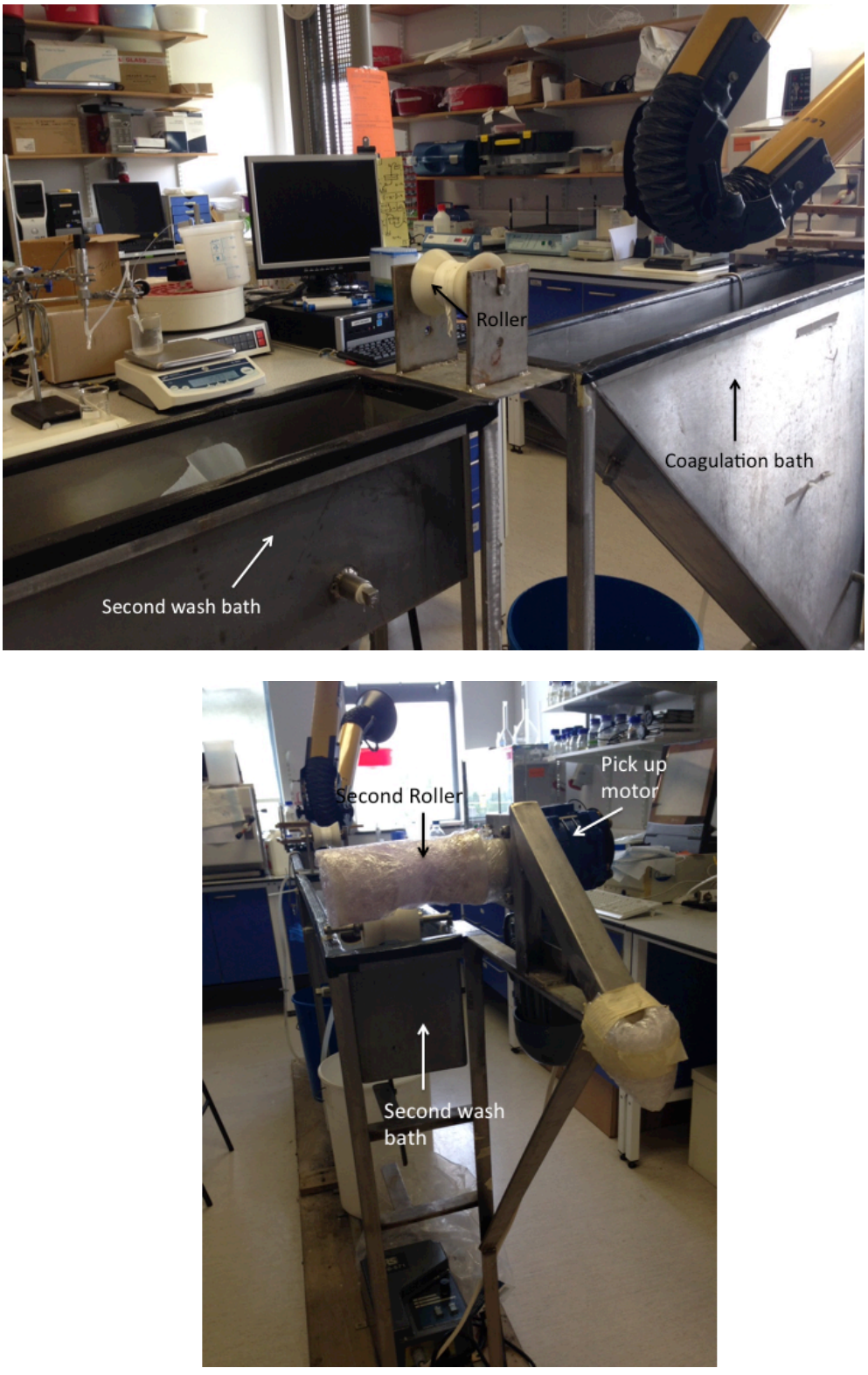


Figure 3.1 – Spinning rig apparatus showing the coagulation water bath and second wash bath with the rollers to guide the fibre formed to the final collection point

The silicon gaskets were sealed on either end of a reactor with silicon glue. Once the gaskets were secured, 2 PLGA fibres were fed into each reactor with the use of tweezers. The void between the fibres in the silicon gaskets at each

end were sealed with further silicon glue. After allowing time for the glue to set, the exposed ends of the PLGA fibres emerging from the reactors were sealed closed with tweezers. Then 5ml of PBS (containing 1% antimicrobial/antifungal solution) was added to each reactor through an open external port using a 5ml syringe and an 18-gauge needle. The open ports of the reactors were closed using silicon tubing attached to one-way valves and kept at 4^{o}C overnight.

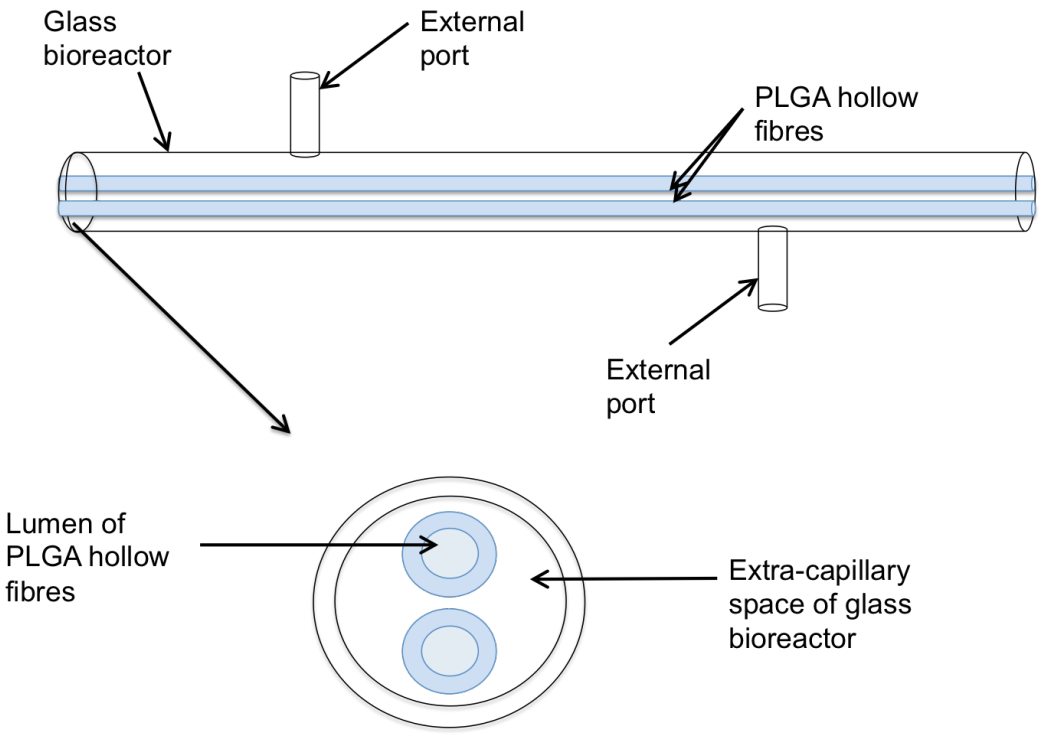


Figure 3.2 Schematic drawing of the Hollow Fibre Bioreactor (HFB) system: side view and cross-section looking into the PLGA fibre lumen and extra-capillary space of the glass bioreactor

Cell culture within PLGA hollow fibre bioreactor system

Cell attachment assays were performed by adding tenocytes at a density of 34,290 cells/cm^2 inside the lumen of the hollow fibres with 5ml of standard culture media. Only tenocytes from early passages (1 to 3) and either from P1 pups or E15.5 foetuses were used. Following the trypsinisation, centrifugation and cell count, as per the method mentioned above, a total of 100,000 cells in 1ml standard culture media was added to an Eppendorf tube. This was done for each fibre per reactor; the cell suspension was centrifuged at 2000 rpm for 5 minutes.

Meanwhile, the PBS (with 1% antimicrobial/antifungal) was removed first, carefully using a syringe and needle via an external port, then the extra capillary space of the glass reactors was injected with 5ml of DMEM while the cell suspensions were prepared. The DMEM from the reactors was removed using a syringe and needle. Following centrifugation of the cells, the supernatant was carefully removed, until 100µl of culture media remained and the cell pellet was resuspended in the 100µl of media and taken up in a 2ml syringe with a 21-gauge needle.

Equal amounts of cell suspension were added internally to the fibres through each end of the reactors. This was done to try and obtain a homogenous distribution of cells on the fibres as possible. Each fibre was slightly longer in length (16cm) than the glass reactors (14cm), so that 1cm of the fibre ends were emerging from each end of the reactors. The fibre ends were sealed using a pair of forceps to force the ends together.

Once the cells had been injected into the fibres, the extra capillary space of the reactors were filled with 5ml of standard culture media using a 5ml syringe and 18 gauge needle. The external ports were sealed using tubing and one-way valves to relieve any pressure trapped within the reactor (Fig. 3.3). The cell seeded reactors were incubated for 24 hours, 3 days and 7 days at 37°C in a humidified 5% CO_2/95% air incubator (as static cultures). Concurrently, controls were done by seeding tenocytes (from foetal and P1 mice) in T25 flasks at the same density of 34,290 cells/cm^2. Additionally, controls were conducted in the HFB system without the cells present.

3.4 Analytical methods

3.4.1 Cell number

Cells were counted using a haemocytometer, together with trypan blue solution to account for cell viability through a light microscope (Eclipse TS100, Nikon). From the cell suspension, a cell sample of 100µl was diluted with 100µl of trypan blue solution. The 9 square grid (3x3) of the haemocytometer is equal to 10^{-4} ml. The total cell count (Equation 1) was calculated as follows:

Equation 1:

$$\frac{cell\ count}{9\ (total\ number\ of\ squares)} \times 2 \times 10,000 \times number\ of\ ml\ of\ cell\ suspension$$

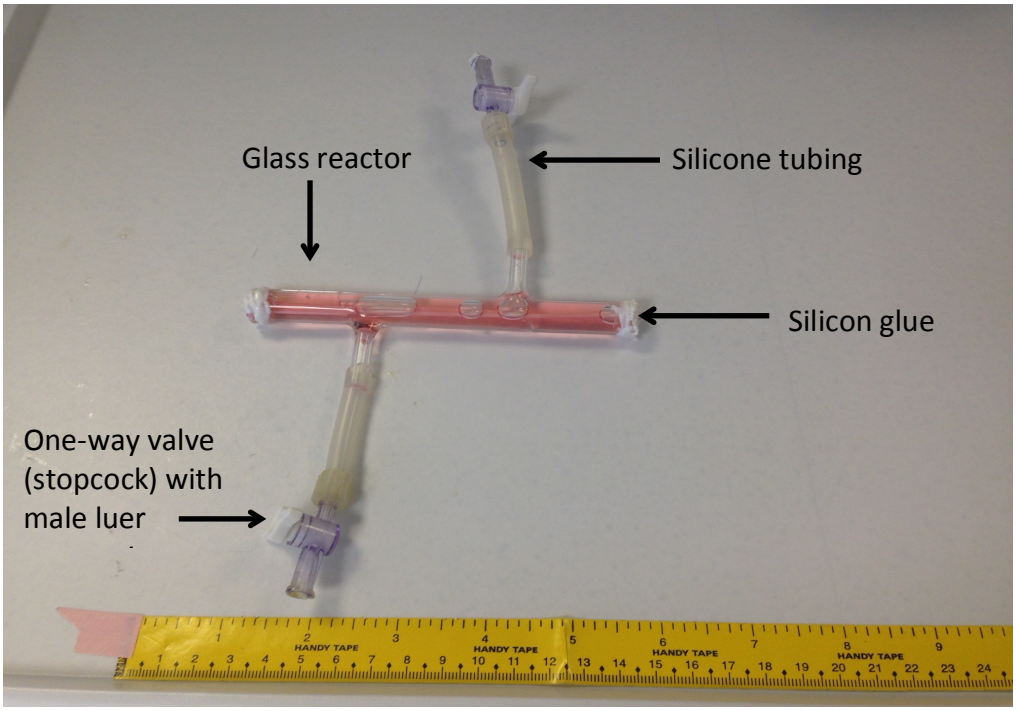


Figure 3.3 Bioreactor set-up showing a control reactor containing cell culture media only.
The silicone glue sealing each end and the use of one-way valves to close off the external ports

3.4.2 Cellular immunostaining

After each cell morphology assay at 6 hours and 24 hours with serum free media and media containing serum, the cells were washed with 2ml of PBS, fixed with 3.7% formaldehyde (Sigma) in PBS for 15 minutes, and permeablised with 400µl 0.5% Triton X-100 (Sigma) in PBS for 10 minutes at room temperature. Next, 200µl diluted Phalloidin-FITC (100µl in 1ml PBS) (Life Technologies) was added to the cells for 40 minutes at room temperature. After further washing of the cells with PBS, the cells were stained with 300µl DAPI nuclear stain (1µl in 50ml in PBS) (Life Technologies) for 5 minutes at room temperature. Finally the cells were viewed using an AMG EVOS inverted fluorescence light microscope (Life Technologies) with a 10x objective.

3.4.3 Cell morphometrics with ImageJ

Using ImageJ (NIH), the area of the cells and circularity were measured to indicate how spread the cells were. Circularity values range from 0.0-1.0 with 1.0 indicating a perfect circle, to a progressively elongated cell shape. Circularity was calculated using the following equation:

$$\text{Equation 2: } Circularity = 4\pi\left(\frac{Area}{Perimeter^2}\right)$$

The process to do this involves several steps on the ImageJ programme to separate the individual cell areas from the background (Fardin et al., 2010) as described and outlined in Figure 3.4. Firstly the quality of the original images were enhanced by adjusting brightness and contrast and converted to 8-bit images, with further enhancement of brightness and contrast. Then the threshold of the images was modified to fragment the cell from the background. Noise on the images was reduced using the tool "Despeckle" which performs a median filter on the image. Then a 2D gradient of each image was obtained using the "Find Edges" tool, which makes use of a Sobel edge detector. The final step uses the "Analyze Particles" tool to count and measure the cells of the threshold images, and an additional step of displaying the results with each measurement can be shown along with outlines of the measured cells overlaid over the contours of the original cell images.

3.4.4 Contact angle measurements

To investigate whether coating the coverslips had any effect on the morphology of the cells; the coverslips were tested to determine their wettability through water contact angle measurements. Briefly, the coverslips were placed in a 6 well plate and pre-coated overnight in 1.5ml PBS or FCS, with one left untreated (as a control). Subsequently the coatings were aspirated off and the coverslips were left to air-dry for 10 minutes.

Contact angle measurements of water were taken at room temperature using the sessile drop method and captured using a contact angle goniometer (Dataphysics Contact Angle System OCA). A drop of 2µl deionisied water was deposited on the surface of each coverslip, via a syringe needle filled with

deionised water (Dataphysics SNS-D051/025 38.1mm length disposable needle) (Fig. 3.5). The experiments were completed in triplicate with each contact angle measurement calculated on the average of 5 pairs of contact angle readings, taken at different points on each sample.

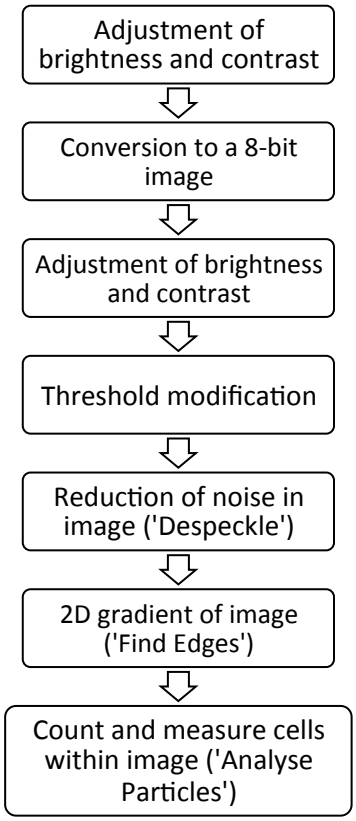


Figure 3.4 The steps to obtaining the quantitative morphometric data of the tenocytes cells from the original images.

3.4.5 Shear stress assays

Tenocytes were trypsinised and passaged as normal and the convergent flow chamber (Fig. 3.6) was seeded with the cells at a cell density of 10,000 cells/cm^2. The cells were added to the chamber through the top port, at the entrance of the chamber. Two vented caps were screwed onto the top two ports and the flow chamber was incubated at 37°C for 3 hours. After the appropriate seeding time, the flow chamber was moved inside a laminar flow hood and attached to a peristaltic pump (Watson Marlow 323) and reservoir with the vented caps removed and replaced with the sealed caps (Fig. 3.6).

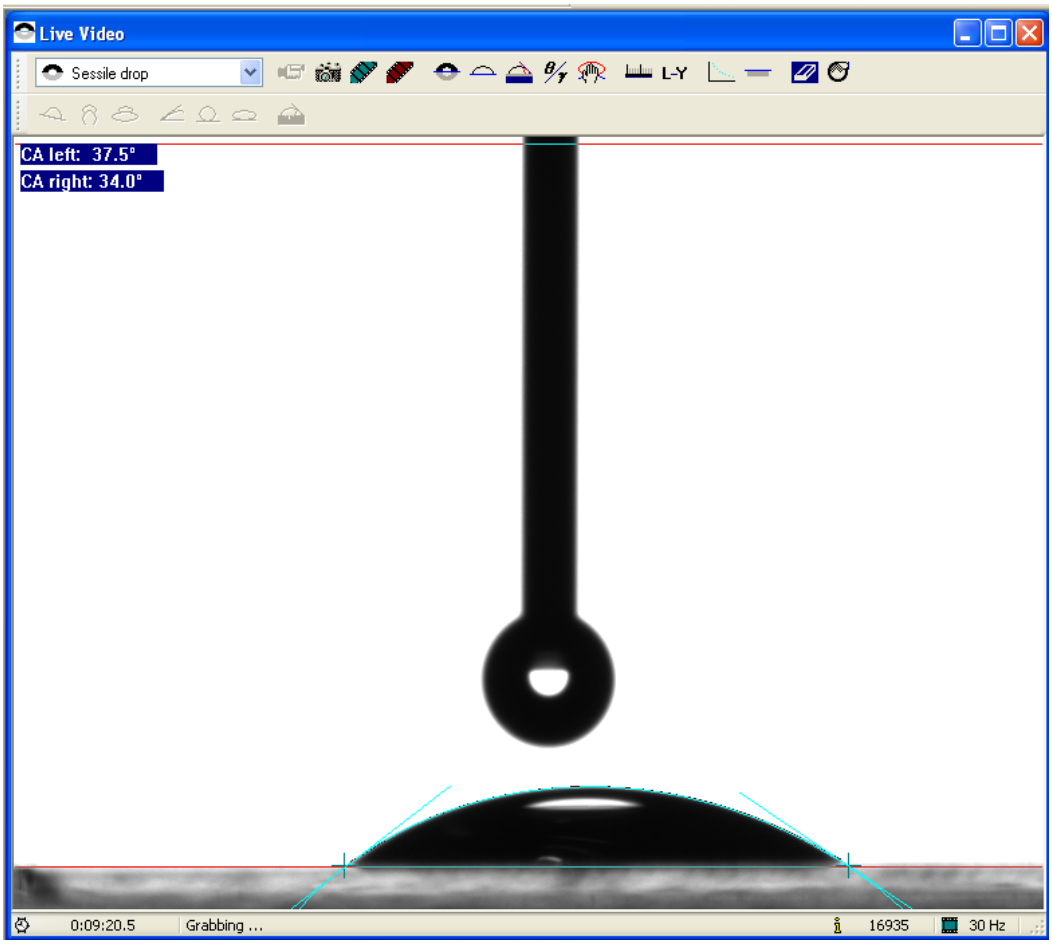


Figure 3.5 Example of water droplet on control glass coverslip surface, showing method of contact angle calculation

The cells were exposed to a preliminary wash in culture medium at a flow rate of 12ml/minute via a peristaltic pump (Watson Marlow 323) for 1 cycle of the pump. Following the wash, 30 images in 3 lines of 10 were taken from the narrow exit end of the chamber to the widest point at 5mm intervals using a Nikon inverted light microscope and camera with the 10mm dark filter at a 10x objective. To provide a control measurement, the flow chamber was then left for a further 15 minutes, before another 30 images were taken of the same positions. The flow chamber was then reattached to the pump and reservoir, and the pump was started at a low shear rate of 10rpm before slowly being increased to the desired flow rate of 66ml/minute for 15 minutes.

The range of shear stress was from 0.102 Pa (1.02 dyne/cm^2) at the narrower end of the chamber to 0.027 Pa (0.27 dyne/cm^2) at the wider end of the chamber. Once the time period had ceased, the pump was stopped and a matching set of 30 images at the same 5mm intervals were taken to compare the effects of shear on the cells. The particular flow rate was chosen as it is close to the shear rate applied to fibroblasts previously, including tenocytes (Mackley et al., 2006).

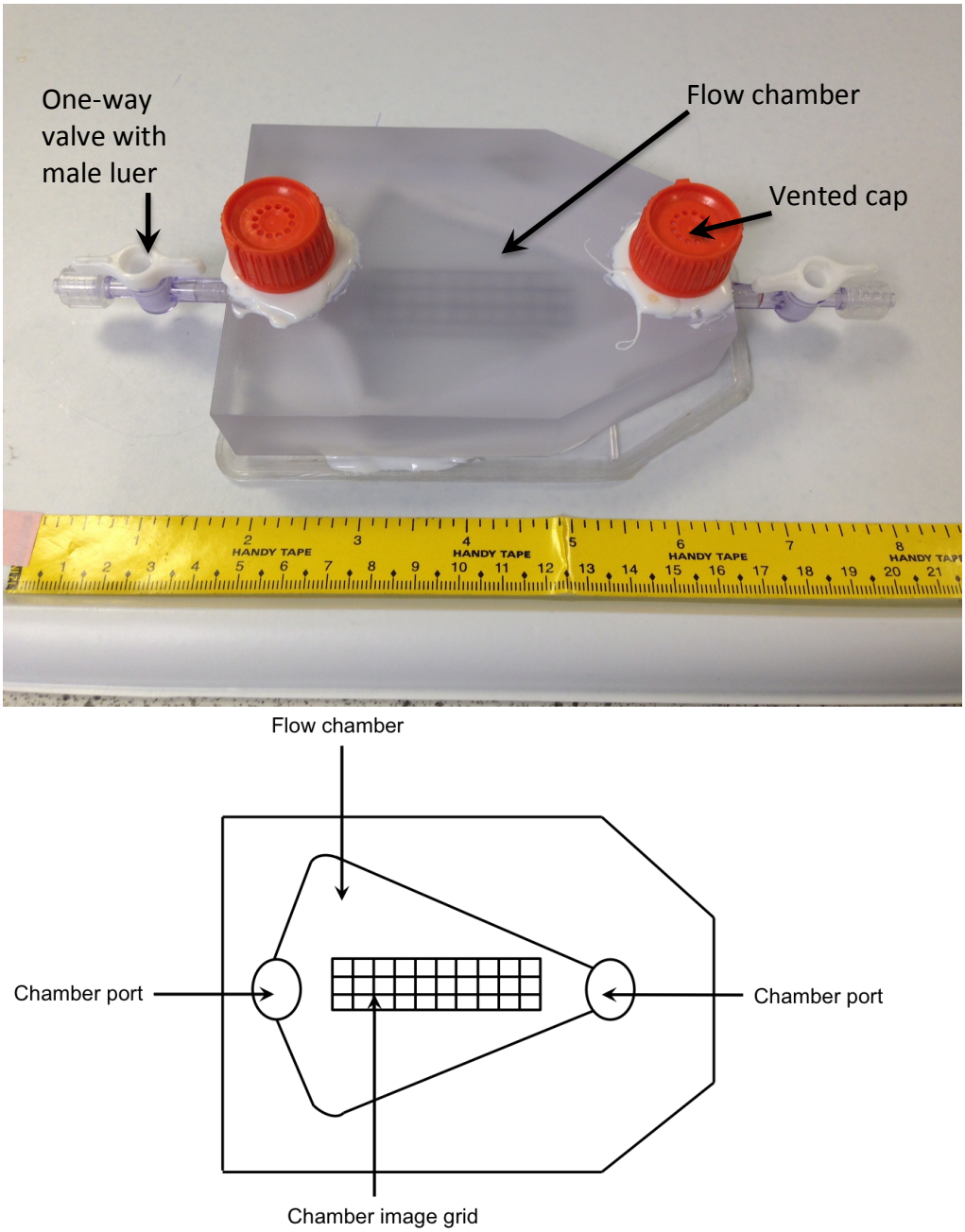


Figure 3.6 Flow chamber shown from the top and schematic illustrating the chamber image grid.

Chamber dimensions: at the narrowest 10mm, at the widest 68.74mm, between the two 66.39 mm, surface area 35 cm^2.

Critical shear stress

The critical shear stress for both the adult and foetal tenocytes was computed, as it was not achieved with either cell type during the experiments. Critical shear stress is defined as the minimum stress needed to detach all cells from the surface of a substrate (Ming et al., 1998). The following established formulae from prior studies was used in order to calculate the critical shear values:

$$\text{Equation 3: } f = 1 - \frac{N_d}{N_o}$$

Where f represents the fraction of cells attached, N_d is the number of detached cells when shear was applied and N_O is the number of initial cells attached before shear was applied.

To support Equation 3 above, Equation 4 can be used to represent the fraction of cells attached in relation to applied shear S, the critical shear value S_C and k which is a dimensionless parameter indicating the degree to which the response digresses from linearity interesting:

$$\text{Equation 4: } f = 1 - \frac{k\frac{S}{S_C}}{k + \left(1 - \frac{S}{S_C}\right)}$$

3.4.6 PLGA hollow fibre characterisation

Scanning Electron Microscopy (SEM)

Sections of the PLGA hollow fibre were prepped for analysis by scanning electron microscopy (SEM). The fibre samples were immersed in liquid nitrogen and cut into 1cm sections and placed on aluminium stubs and kept in place by double-sided carbon charging tape. The samples were then gold sputter coated (Edwards S150B Sputter Coater) and ready for evaluation using the JEOL SEM 6480LV. Images were taken of the fibre lumen under vacuum and surface at a working distance of 20.0mm and beam accelerating voltage of 2.0 kV.

Tensile testing

Samples of the PLGA hollow fibre were characterised for their mechanical properties through tensile testing. Tensile testing was done at the Advanced Composites Centre for Innovation and Science (ACCIS) at the University of Bristol where Dr Sameer Rahaketar kindly provided access to use of an Instron 3343 machine and Bluehill Software to collect the data. Samples were prepared by cutting the fibre into 2cm sections and individual samples were loaded into the machine and secured before a load was applied. A loading cell of 10N was applied at a rate of 1.5mm/min.

3.4.7 PicoGreen

Upon the end of cell attachment, the reactors were disassembled for the cells to be prepared for analysis using a Quant-iT PicoGreen dsDNA assay kit (Fisher). Each fibre was cut into 2cm sections and immersed in 1ml of TE buffer diluted in PBS (1ml TE buffer in 19ml PBS) in individually labelled 1.5ml Eppendorf tubes. The samples were then stored at -80^{o}C, before being subjected to 2 freeze-thaw cycles before conducting the PicoGreen assay.

The λ DNA standard was made up at concentrations from 1500ng/ml to 0ng/ml in diluted TE buffer and used to ensure the PicoGreen dye was working effectively (Table 3.7). A cell standard was also used to create a standard curve to compare the experimental sample values against. Using a 96 well plate, the cell standard occupied wells A1 to H4, with 4 wells for each dilution of the cell standard starting with 9000 cells in 75µl of diluted TE buffer in Row A, to 0 cells in 75µl of diluted TE buffer (Table 3.8). 75µl of diluted PicoGreen dye (50µl of PicoGreen dye in 9.95ml of diluted TE buffer) was added to each well of the cell standard.

Table 3.7 Concentrations and volumes of PicoGreen λ DNA standard

Λ DNA standard volume (µl)	**TE buffer volume (µl)**	**Concentration (ng/ml)**
5	995	1500
667 of above	333	1000

500 of above	500	500
250 of above	750	125
250 of above	750	31.25
250 of above	750	7.8125
250 of above	750	1.953
0	1000	0

Table 3.8 Dilution and volumes of cell standard for PicoGreen assay

Number of cells in 75 µl	Number of cells/ml	TE buffer (µl)
(Starts with 18,000) 9,000	120,000	75
4,500	60,000	75
2,250	30,000	75
1,125	15,000	75
562,5	7,500	75
281,25	3,750	75
140,625	1,875	75
0	0	75

The remaining wells are occupied by 75µl of each cell sample from the fibre segments, in quadruplicate with 75µl of diluted PicoGreen per well. The well plate was read on a BioTek Synergy HT Fluorospectrometer, with the corresponding Gen 5 software programme with the plate layout entered in advance. Using the cell standard samples fluorescent intensity readings corresponded to a known number of cells and a standard curve was compiled.

3.4.8 Metabolic assays

Media from the proliferation experiments in the HFB systems, along with media from the controls on tissue culture plastic (TCP) and within the reactors, was retained at the end of each time course experiment to examine cell metabolic activity. This was done namely in the form of glucose consumption and lactate production.

Glucose assay

Glucose metabolism assays were done using a MegaZyme D-Glucose GOPOD kit K-GLUC, with standard culture media used as the standard. DMEM (Glutamax) glucose content is 4.5g/l. Therefore, 10µl = 45µg of D-glucose. The Glucose Determination Reagent (GOPOD reagent) was made up into 10ml aliquots and stored at -20°C and thawed out once for the assay. The samples and media aliquots were also thawed out from storage in the -80°C freezer. In 1.5ml Eppendorf tubes, the reagent blank, standard and samples were prepared (Table 3.9). The tubes were then incubated on a hotblock at 50°C for 20 minutes, before 280µl from each tube were aliquoted out into a 96 well plate in triplicate. The plate was finally read on a BioTek Synergy HT Fluorospectrometer at a wavelength of 510nm. To calculate the glucose content, the average reading for the reagent blank was subtracted from the sample readings and media standard readings. The D-Glucose measurement was calculated using the following equation:

Equation 5: $D-Glucose\ (\mu l/0.1ml) = \frac{\Delta A_{Sample}}{\Delta A_{D-Glucose\ standard\ (100\mu g)}} x100$

Table 3.9 Dilutions and volumes of samples, standard and reagent blank for glucose assay

	Reagent blank	Standard	Sample
GOPOD reagent	1ml	1ml	1ml
D-Glucose standard (media)	-	10µl	-
Sample	-	-	10µl

ddH_2O	10µl	-	-

L-lactate assay

L-lactate assays were also performed using MegaZyme L-lactic acid kit K-LATE. Firstly, the standard was prepared (Table 3.10). 10µl of the standard dilutions were added in duplicate to a 96 well plate. The samples, controls and media were diluted in ddH_2O (1 in 5 dilution) in the same 96 well plate and stored at -20°C overnight if required. The remaining reagents were then combined together (Table 3.11) and 212µl of the reagent mix was added to each well containing 10µl sample/standard in duplicate. The plate was read on a BioTek Synergy HT Fluorospectrometer at a 340nm wavelength (A_1). The reaction was started with the addition of 1.7µl of L-LDH per well. The plate was read again at a 340nm wavelength every 3 minutes from the start of the reaction (0 min) to an endpoint of 27 minutes.

The difference in absorbance for both the blank and experimental sample was verified using the absorbance value after 10 minutes (A_2) and initial absorbance value (A_1). The concentration was calculated using the following formula in order to obtain the change in absorbance of L-lactic acid ($\Delta A_{\text{L-lactic acid}}$) which was determined by subtracting the absorbance difference of the blank from the difference of the sample:

$$\text{Equation 5: } c = \frac{V \; x \; MW}{\varepsilon \; x \; d \; x \; v} \; x \; \Delta A_{L-lactic\ acid} \quad (\text{g/L})$$

Where V = final volume (ml), MW = molecular weight of L-lactic acid (g/mol), ε = extinction coefficient of NADH at 340nm (6300), d = light path (cm) and v = sample volume (ml) c = concentration of L-lactic acid. For L-lactic acid this follows as: 0.3204 x $\Delta A_{\text{L-lactic acid}}$.

Table 3.10 Dilutions and volumes for assay standard

Buffer 5 (L-lactic acid - 150µg/ml)	**ddH_2O**	**L-lactic acid in 10µl**

20µl	-	3µg (in 20µl)
10µl	-	1.5µg
24µl	24µl	0.75µg
24µl of above	24µl	0.375µg
24µl of above	24µl	0.1875µg
24µl of above	24µl	0.09375µg
-	10µl	0µg

Table 3.11 Volume of reagents added to each well

	Volume per well (µl)	Final total volume per well (µl)
ddH_2O	150	212
Buffer with D-glutamate and sodium azide (0.02%)	50	
NAD^+ + PVP	10	
D-Glutamate-pyruvate transaminase (GTP) suspension	2	

3.5 Statistical methods

3.5.1 Data representation

Data was plotted from calculated average values with error bars representing ± standard error. The number of samples per experiment varied and is therefore stated within the caption of each figure. Each experiment was repeated in triplicate as a minimum, and more in particular instances.

3.5.2 Comparative statistics

One-way ANOVA (one way analysis of variance) together with Tukey's post hoc tests were used to determine the statistical significance ($P<0.05$) between independent samples done at minimum in triplicate.

Chapter 4 – Development and optimisation of a method for tendon cell isolation

4.1 Introduction

A suitable number of primary adult and foetal tenocytes were essential to studying the characterisation of the cells and later their proliferation within hollow fibre bioreactors (HFB's). Ultimately the cells would be utilised within a clinical setting for repair of a patient's tendon injury. Therefore, having an appropriate technique to obtain an initial population of cells that can be expanded *in vitro* is required. The aim here was to thus compare two cell isolation methods for the highest number of cells in terms of confluence.

In contrast to many other cell types, tenocytes are not available to use through a cell line. Furthermore, in adults, tendon tissue predominantly consists of extracellular matrix (ECM) and the cell population is relatively low. In contrast, foetal tendon tissue has a higher cell population and the ECM constitutes much less of the overall tissue content (Dahners, 2005; Stoll et al., 2010). Therefore, isolation of tenocytes from primary tissue is possible, via either outgrowth of cells from the tissue (primary explant culture) or enzymatic digestion of tissue using proteolytic enzymes. It was hypothesised that the explant culture would be an inadequate technique for isolation of adult tenocytes in comparison to enzymatic digestion.

Primary explant culture as a technique of isolating cells from tissue has existed for over a century, with the basic protocol remaining virtually unaltered (Freshney, 2011). Cell outgrowth occurs at a faster rate from foetal explant tissue than adult explant tissue, and the cell yield from foetal tissue is higher than adult tissue (Lanza et al., 2011). However, the use of enzymes, principally trypsin/EDTA, collagenase and dispase allows a cell suspension to be gained at a much faster rate than explant culture.

The use of a specific enzyme is reliant upon on the tissue being used and the cell adhesion molecules (CAMs) present. Trypsin is combined with EDTA, as in

its pure form, it has a poor affinity for extracellular proteins. EDTA serves as an effective chelating agent on calcium sensitive CAMs (Freshney, 2011). Both techniques of explant culture and enzyme digestion were explored as methods to isolate tenocyte populations from the primary tissue.

4.1.1 Revisions to primary explant technique

To improve the rate of cell proliferation and cell yield, the primary explant technique underwent two separate variations in order to achieve at least 70% cell confluence at a faster rate. The first of these was the inclusion of a glass coverslip (Chard et al., 1987), which was placed on top of the pieces of explant tissue, and secondly scoring the surface of individual wells within a well plate before placing the explant tissue pieces on top of the scratches to attach (Even-Ram and Artym, 2009). Both these methods were introduced to enhance the attachment of the explant tissue to the surface of the well plates and allow cell migration from the tissue.

4.1.2 Enzymatic digestion of tendon tissue

Multiple steps in the cell isolation protocol were further changed to replace explant culture by employing the use of various enzymatic digestion methods, namely a combination of collagenase and dispase. These enzymes have previously been used on tendon tissue to release cells quickly for proliferation (Bi et al., 2007; Jiang et al., 2014b; Ruzzini et al., 2013; Theiss et al., 2015). Additionally, using trypsin/EDTA to digest tissue has also been proven effective (Freshney, 2011) at making cells rapidly available for subsequent expansion. Therefore, a minimum of 70% cell confluence was attained more promptly than was possible with explant culture.

4.2 Results

4.2.1 Cell culture with primary explant technique

Glass coverslips

Explants of adult and foetal murine tendon tissue were cultured with glass coverslips to secure the tissue within 6 well plates. Initially, observed outgrowth of cells from the tissue was good, with cell outgrowth from the foetal tendon tissue occurring at a faster rate than the adult tendon tissue, with foetal tenocytes on average having migrated out from the tissue within less than 2 weeks. The average time to reach confluence (approx. 70%-80%) for adult tenocytes, was more than twice as long using the same technique (Figure 4.1). To improve upon the length of time to confluence for adult tenocytes, a second method was trialled and detailed below.

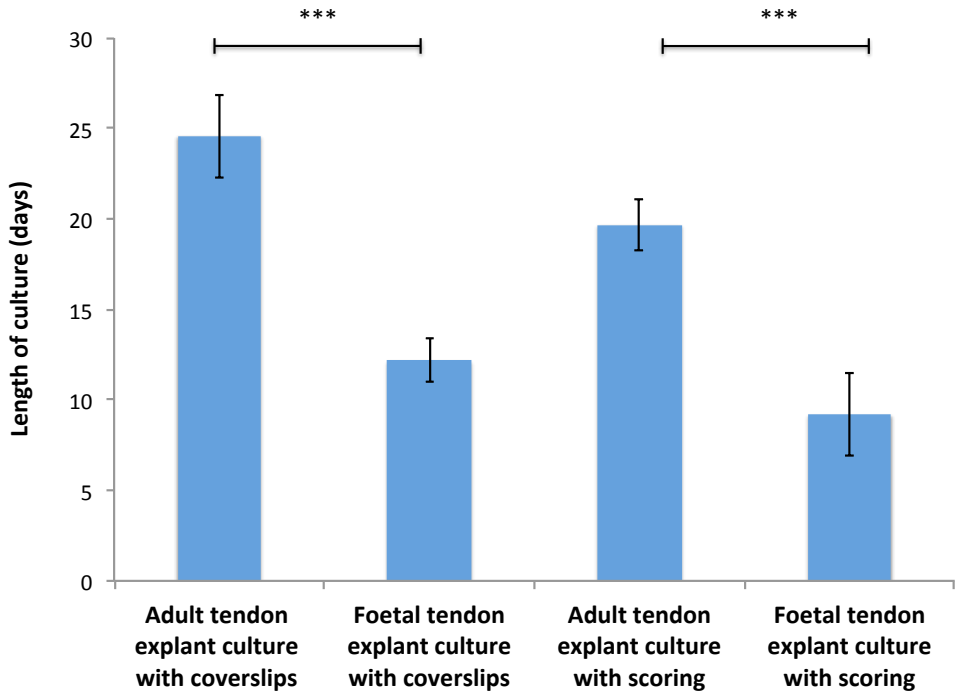


Figure 4.1 Average length of time to confluence for adult and foetal tenocytes with the explant technique.
Quantitative data was obtained to compare the time taken for cells to reach confluence (70-80%) using the explant technique with either glass coverslips n = 3, N = 12 (n = sample size, N= population size) or scoring the bottom of well plates n = 3, N = 9 (*** = $p < 0.001$) Error bars are ±SEM.

Scoring well plate

Scoring the surface of the well plates resulted in a slight decrease in the length of time to confluence for adult tenocytes. Similar to using glass coverslips, scoring the bottom of the well plates was done to encourage better attachment of the explant tissue to the surface of plates. Though the difference was not found to be statistically significant (Figure 4.1). This reflects that this particular method for culture became stagnant with little change in adult tenocyte outgrowth; hence the subsequent investigations into conducting cell culture with enzymes.

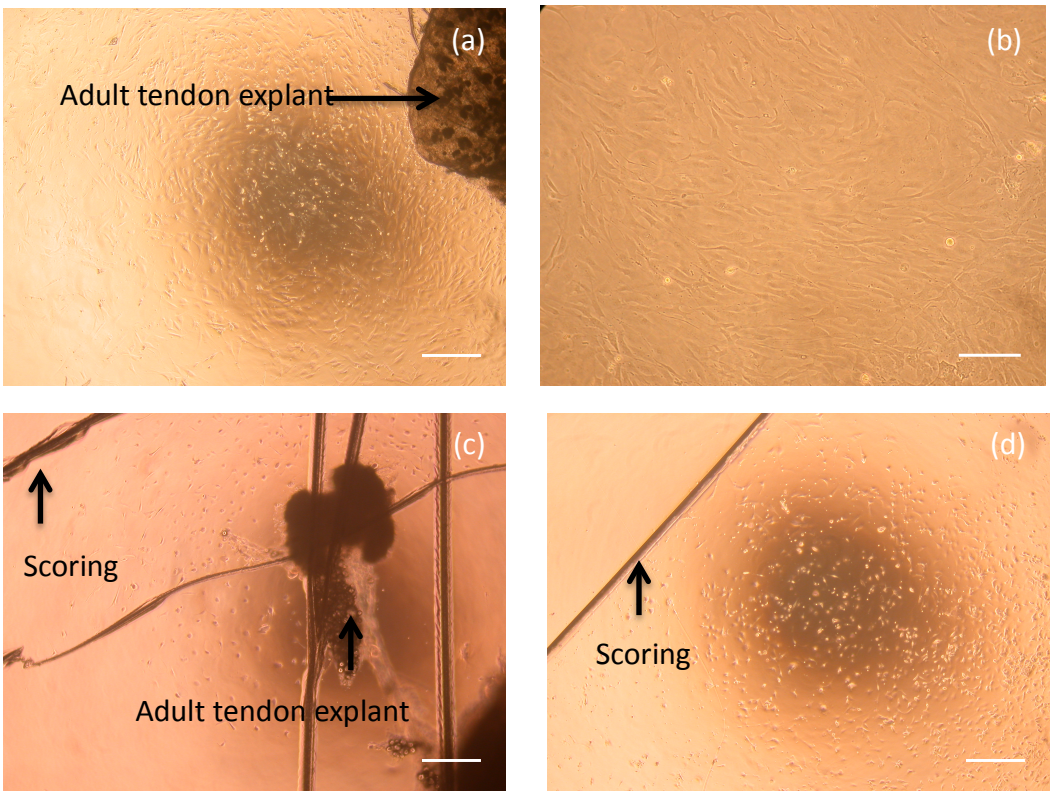


Figure 4.2 Outgrowth of tenocytes from adult and foetal tendon tissue with the different explant techniques.
(a) With adult tendon using a glass coverslip at Day 22 of culture. (b) With foetal tendon using a glass coverslip at Day 14. Scale bar represents 240µm at 10x magnification. (c) With adult tendon using scoring at Day 22 of culture. (d) With foetal tendon using scoring at Day 7 of culture. Scale bar represents 530µm at 4x magnification (a, c and d) and 240µm at 10x magnification for (b).

4.2.2 Cell culture with enzymatic digestion of tendon tissue

With collagenase and dispase enzymes

Firstly, the use of collagenase type IV and dispase were used in combination together to digest the tendon tissue and make the cells available immediately for culture. They have previously been employed for use with tendon tissue and proven to be effective (Bi et al. 2007; Ruzzini et al. 2013; Jiang et al. 2014; Theiss et al. 2015). The use of collagenase type IV and dispase meant that the adult tenocytes were able to reach confluence within a substantially shorter period of time (Figure 4.3), bringing the average length of adult tenocyte culture to less than 2 weeks and within the same range as the time taken for the foetal tenocytes to reach confluence, with the same technique.

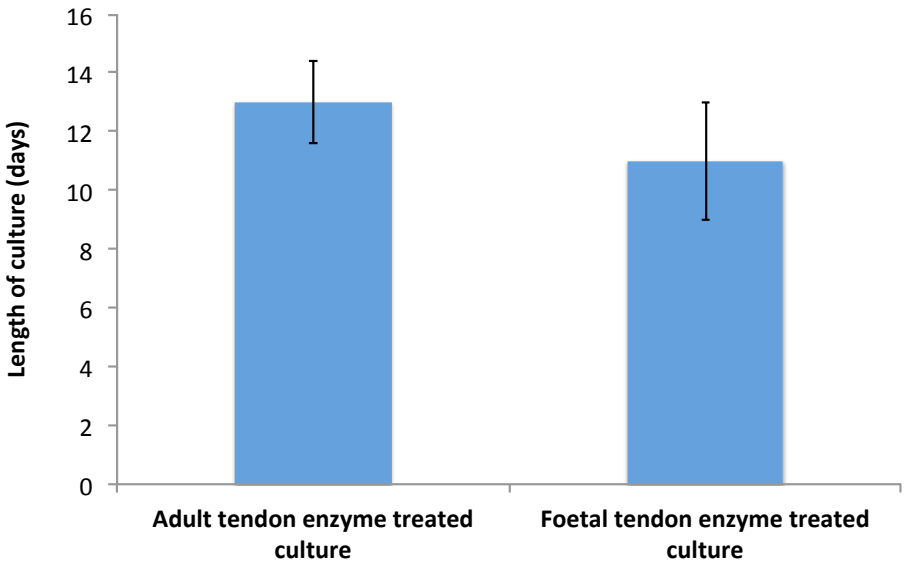


Figure 4.3 Average length of culture time for tenocytes with enzyme treatment of adult and foetal tendon tissue.
Quantitative data was obtained to compare the time taken for adult and foetal tenocytes to proliferate using enzyme treatment of tendon tissue n = 3, N = 10.

With addition of cell strainer

With the inclusion of a cell strainer post enzymatic treatment of tissue, interestingly there was no observed improvement in the number of both adult and foetal tenocytes that were recovered, as demonstrated by the large fluctuation in the number of cells counted (Figure 4.4) with and without the use

of a cell strainer. Due to this finding, all future tenocyte isolations from the tissue were conducted without them.

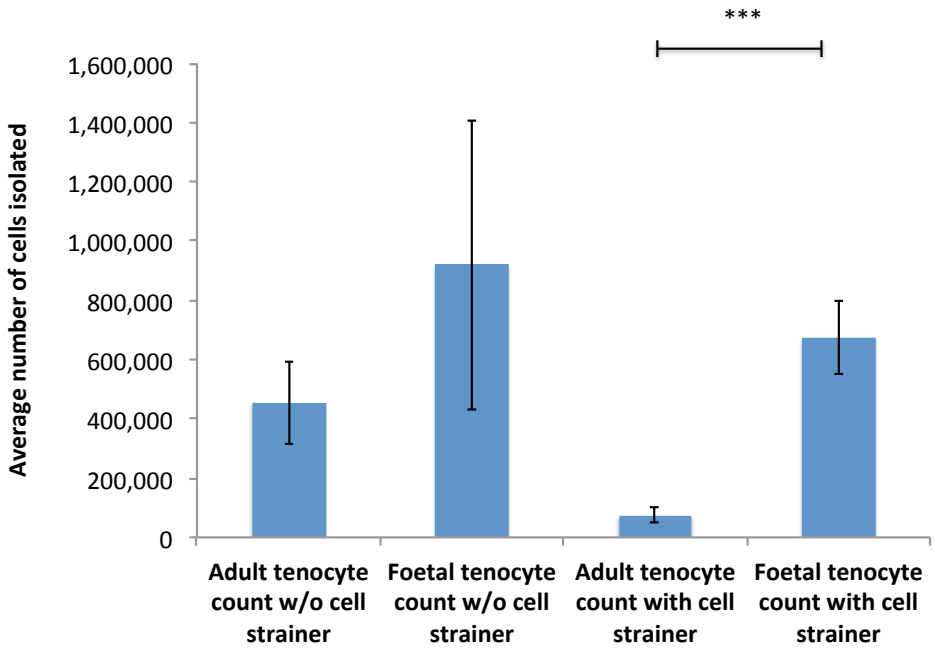


Figure 4.4 Average number of adult and foetal tenocytes isolated with a cell strainer after enzyme digestion of tendon tissue.
Quantitative data was obtained to compare the number of cells acquired with a cell strainer (n=6, N=1) and without a cell strainer (n=16, N=1) (*** = $p < 0.001$) Error bars are ±SEM.

4.3 Discussion

4.3.1 Cell culture with primary explant technique

The aim of this work was to discover a successful method to extract the largest number of adult and foetal tenocytes from primary tendon tissue, especially for adult tenocytes given the unique composition of adult tendon tissue. As mentioned above, explant culture is a well-established method of extracting cells from primary tissue. It has been used successfully to isolate cells from tendon tissue previously (Freshney, 2011). However, the extent of time required for adult tendon explant culture reveals the necessary time for the tenocytes to begin replicating, and secondly it demonstrates the slow migration of the cells out from the dense connective tissue; which in adults has been highlighted as consisting mainly of ECM (Fu et al., 2008; Ruzzini et al., 2013; Stoll et al., 2010). Higher levels of collagen content have been identified in adult

rat tendon, resulting in stiffer tissue (Kular et al., 2014). Tenocytes occupy the space between the collagen fibres of tendon tissue (Ruzzini et al., 2013). Therefore, if in adult tissue there is a higher ECM content in comparison to cells then this makes it difficult to successfully extract cells using explant culture. Applying the separate use of glass coverslips and scoring the surface of the well plates has the effect of increasing the surface area between the tendon tissue and underlying substrate surface (Chard et al., 1987; Even-Ram and Artym, 2009).

Explant culture has some advantages including being the least damaging way of isolating primary cells from tissue, with the 3D structure of the original tissue being preserved (Freshney 2011). Hence, more viable cells are obtained, at the expense of a lower cell yield, in comparison to using enzymatic methods (Stacey, 2005). The tenocytes attained via explant culture also have the advantage of being likely to share similarities with tenocytes *in vivo* (Fu et al., 2008), for example cell behaviour will likely reflect the behaviour of the tenocytes within the tissue. The results demonstrated that this technique was more successful with the foetal tendon tissue, however this tissue is known to be more cellular and adult tenocytes proliferate at a slower rate *in vitro* with cells taking up to 4 weeks to reach confluence (Abousleiman, 2008; Chard et al., 1987).

Evidently, using explant culture to harvest tenocytes, in particular adult tenocytes was too lengthy a process, despite the method being widely used and reported to take a much shorter span of time (Chen et al., 2008; Schulze-Tanzil et al., 2004; Tohidnezhad et al., 2011); whilst other studies fail to report on how long primary explant culture took before cells were confluent (Chao et al., 2008; Lavagnino and Arnoczky, 2005). Therefore based on the results obtained, explant culture should not be used as a recommended technique to cultivate tenocytes.

4.3.2 Cell culture with enzymatic digestion of tendon tissue

Obtaining a higher yield of cells is easier to achieve with the use of enzymatic digestion of the original tissue (Wagenhäuser et al., 2012) often with a significant difference in the number of cells attained. Furthermore, there is a significant reduction in the length of culture time, reflecting the action of the enzymes in successfully breaking down the tissue to release the cells immediately for proliferation. However, despite the improvement in quality and yield of the cells with enzymatic digestion, the method requires particular attention by monitoring the prime temperature necessary together with the length of time for exposure of the tissue to the enzyme treatment. Without careful control of these two particular parameters, the cell viability can be compromised (Stacey, 2005).

The variable results produced with the additional step of using cell strainers, questions the effectiveness of using them following enzymatic treatment on tendon cells. There are several examples where cell strainers have been utilised successfully to isolate tendon cells from digested tissue (Brown et al., 2014; Mazzocca et al., 2012; Mienaltowski et al., 2012; Theiss et al., 2015). Nevertheless, in each of these circumstances smaller size mesh cell strainers were used, being either 70µm or 40µm mesh cell strainers. Therefore, this indicates that the appropriate sized cell strainers could be employed alongside enzyme dissociation to isolate a higher yield of tenocytes.

This difference in isolation methods has also been reported as causing changes in cell characteristics such as cell proliferation and cell morphology (Xiao-tao et al., 2010). In order to achieve positive outcomes for tendon tissue engineering, cell proliferation arguably has a critical part to play (Declercq et al., 2004). This is mainly due to the low number of initial cells present within primary tissue (Shen et al., 2013), which presents an immediate challenge to expansion of cell numbers *in vitro*. Additionally age has been acknowledged as having detrimental impact on the proliferative capabilities of primary tendon cells (Bayer et al., 2012).

Secondly due to the phenotypic drift that tenocytes will eventually experience, the number of subcultures tenocytes can undergo for use in *in vitro* studies is a limiting factor (Mazzocca et al., 2012). It could also be argued that by expanding primary tenocytes within the first few passages, phenotypic drift can be mitigated, whilst also maintaining a phenotype that closely matches *in vivo* tenocytes (Yao et al., 2006).

4.3.3 Alternative tendon cell sources

The problems encountered when attempting to successfully isolate and culture primary tenocytes, could explain why dermal fibroblasts have commonly been used in preference to tendon fibroblasts in previous studies that have looked to examine tendon. This is primarily due to their ease of isolation, faster proliferation rate and comparable behaviour to tendon fibroblasts (Brink et al., 2009; Tang et al., 2014). Other studies (Bullough et al., 2008; Morita et al., 2012; Thaker and Sharma, 2012) have preferred to use mesenchymal stem cells (MSCs) to investigate repair and regeneration of tendon injuries. Again, using stem cells has several advantages over tenocytes taken from explanted tissue, including their pluripotency and higher proliferative ability. The type of the isolation technique used has been shown to influence the expression of particular tendon genetic markers in MSCs; with an increase in the levels of scleraxis when MSCs were isolated using enzyme digestion, in comparison to those isolated via explant culture. (Gittel et al., 2013) The explanation for this was suggested as the tissue is broken down, collagen products are formed which activates the escalation in scleraxis expression; this process replicates the initial steps of healing and thereby promotes the surge in tendon marker expression.

The recent discovery of tendon stem/progenitor cells (Bi et al., 2007; Mienaltowski et al., 2012) has resulted in these particular cells being investigated as a source for cell expansion *in vitro* with the application of being used to heal tendon injuries *in vivo* through the process of differentiation towards mature tenocytes (Jiang, et al. 2014; Zhou et al. 2010; Ruzzini et al. 2013). This is possible due to the tendon stem/progenitor cells positively

expressing genes that are linked to tendon such as scleraxis and tenomodulin. Therefore, they could be a better potential cell source than the conventional option of mesenchymal stem cells.

Despite this promising finding, distinct age related differences have been identified between tendon stem cells extracted from young and aged sources (Kohler et al., 2013). Some of these differences include a lower cell population and alternations in cell morphology, which are associated with changes in the actin cytoskeleton (this will be explored in further detail in the next chapter). Therefore, even when considering the use of tendon stem cells, the primary source has to be taken into account, with a young or foetal cell population being the more favoured option.

4.4 Conclusion

Considering that the end application for the tenocytes would be within a clinical setting to repair a patient's tendon injury, having a reproducible and reliable isolation technique to obtain the initial pool of cells is a critically important. The two main methods of explant culture and enzyme digestion trialled each had their advantages and disadvantages. For explant culture the main positives were that the method is less invasive and potentially damaging to the cells, together with the fact the natural surrounding tissue was still present providing a similar environment to the one found *in vivo*. Furthermore, explant culture is cheaper as it does not require the use of enzymes. However, the number of cells that can be successfully isolated using explant culture was very poor and could only be judged on a qualitative basis and the culture time was significantly longer for adult tendon than foetal tendon. These two factors combined make explant culture an unlikely choice within a clinical setting.

In contrast, using the method of enzymatic digestion can adversely affect cellular properties due to the breakdown of the natural tissue environment. In addition to this the required use of enzymes makes this method more expensive. Nevertheless, the number of cells attained could be measured

quantitatively and there was a significant reduction in the length of culture time for the adult tenocytes observed. This can be explained by the fact the enzymes work to release the cells immediately for culture. To conclude, enzymatic digestion was chosen as the preferred cell isolation technique due to the higher yields of cells, particularly adult tenocytes that could be acquired in a shorter culture time.

Chapter 5 – Characterisation of generation-dependent variation in tendon cell morphology

5.1 Introduction

Once a successful method of isolating the adult and foetal tenocytes had been established, the next objective was to characterise age-related phenotypic variations between adult and foetal tenocytes, and also explore the links between material surface properties, protein adsorption; with the aim of investigating how these factors may affect cell attachment, spreading and the cytoskeleton. There has been limited research at the cell-matrix level that has not concentrated at examining the differences from foetal and adult sources, with previous focus upon dermal fibroblasts in particular (Brink et al., 2009; Tang et al., 2014). Despite this, there is virtually no research on comparing the phenotypes of adult and foetal tenocytes. It was hypothesised that foetal tenocytes would display characteristics independent of external stimuli, such as substrate surface chemistry, and in comparison the adult tenocytes would be more sensitive to these factors.

5.2 Results

5.2.1 Contact angle of the different substrates

All glass substrates were shown to have wettability within the range to promote cell spreading i.e. hydrophilic with a water contact angle (WCA) value below 90° (Oliveira et al. 2014). However, there was a significant difference in the level of hydrophilicity, between the substrates (Fig. 5.1). Using the water contact angle (WCA) to determine the wettability, the control coverslip already had a hydrophilic surface (between 90°-10°). With the addition of pre-coating the coverslips the degree of hydrophilicity increased, with the serum treatment of the surface increasing the hydrophilicity significantly than the control coverslips (WCA below 18°), whilst the coverslip pre-treated in PBS had a superhydrophilic surface (WCA below 10°) (Oliveira et al. 2014).

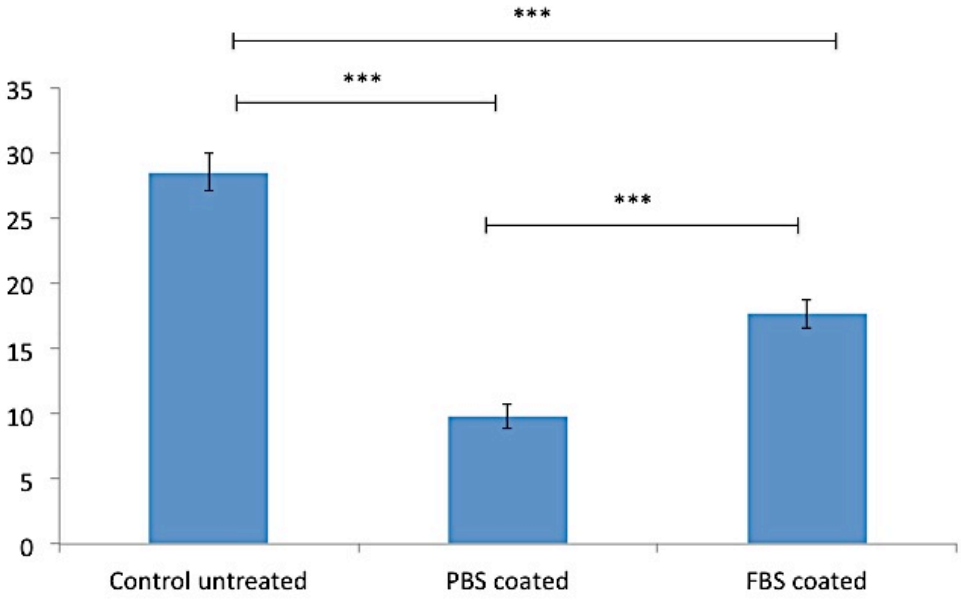


Figure 5.1 – Average contact angle measurement for each substrate coating
Excluding the controls, coverslips were pre-coated with PBS or FBS overnight. Subsequently the coatings were aspirated off and the coverslips were left to air-dry for 10 minutes. Contact angle measurements of water were taken at room temperature using the sessile drop method and captured using a contact angle goniometer. Each contact angle measurement was calculated on the average of 5 pairs of contact angle readings, taken at different points on each sample n = 3 N = 5 (*** = $p < 0.001$) Error bars are ±SEM.

5.2.2 Cell morphology on different substrates

The morphology of the adult and foetal tenocytes was examined after 6 hours and 24 hours under serum-containing media and serum free media to provide a comparison of how proteins are presented (in suspension or as a substrate coating) to the cells might influence the cells to change their phenotypic behaviour.

Serum-containing media

Overall the adult tenocytes became gradually more elongated and larger with more time (Fig. 5.2 and Fig. 5.3), with this becoming more evident when the cells were seeded onto coverslips adsorbed in serum (Fig. 5.2c and 5.3c). In contrast, the foetal tenocytes remained almost unchanged in both their size and shape, after both 6hrs and 24hrs of attachment (Fig. 5.4a and 5.4b). There was no significant difference compared to the control substrate of cell morphologies

in the presence of serum for the foetal tenocytes, with the size of the cells remaining almost unaffected under the different conditions and their circularity also reflected this, with little change in how spread the foetal tenocytes were. However serum made a significant difference to the shape of the adult tenocytes (Fig. 5.4a and 5.4b) with the adult tenocytes becoming larger in size and more elongated in shape, especially after 24 hours attachment.

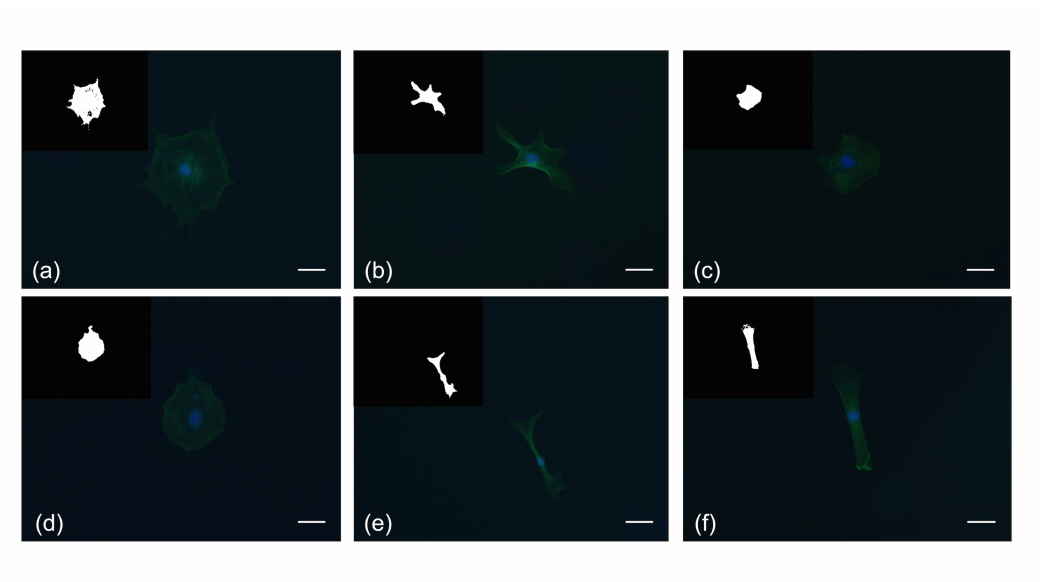


Figure 5.2 Tenocyte cell morphology after 6hrs attachment of actin cytoskeleton (green) and cell nucleus (blue) at 10x magnification with cell outline inserts.
Adult and foetal tenocytes were seeded onto glass coverslips at 5,000 cells/cm2 then fixed, permeabilised and immunostained with FITC-phalloidin, imaged at 10x and assessed using ImageJ: (a) adult tenocyte on a untreated coverslip, (b) adult tenocyte on a coverslip adsorbed in PBS, (c) adult on a coverslip adsorbed in FBS, (d) foetal tenocyte on an untreated coverslip, (e) foetal tenocyte on a coverslip adsorbed in PBS, (f) foetal tenocyte on a coverslip adsorbed in FBS. Scale bar represents 50 µm.

Serum free media

After 6 hours of culture in serum free media, the foetal tenocytes showed there was little change in the cell morphology (Fig. 5.5 and Fig. 5.7a), even with the presence of a serum coated substrate. The adult tenocytes remained almost unchanged when there was no serum present under control conditions and on a PBS coated substrate. However, in the presence of a serum coated substrate, the adult tenocytes morphology was more closely aligned to the morphology of the foetal tenocytes. With the adult tenocytes becoming more rounded on the serum coated substrate, although under all 3 conditions the adult tenocytes

were still larger in size than the foetal tenocytes (Fig. 5.7a). Exposing the foetal tenocytes to a serum coated surface for 24 hours resulted in the cells responding by altering their morphology, by becoming more spindle like in their shape as their circularity fell and becoming larger in size. This closely follows the behaviour mirrored in the adult tenocytes under the same conditions (Fig. 5.7b).

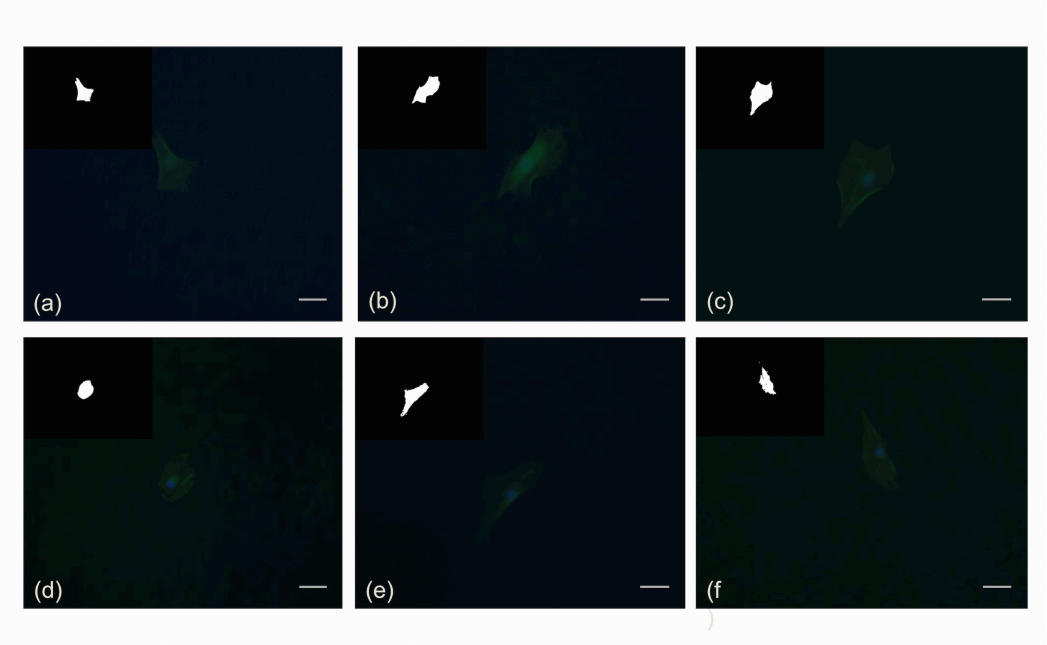


Figure 5.3 Tenocyte cell morphology after 24hrs attachment of actin cytoskeleton (green) and cell nucleus (blue) at 10x magnification with cell outline inserts
Adult and foetal tenocytes were seeded onto glass coverslips at 5,000 cells/cm^2 then fixed, permeabilised and immunostained with FITC-phalloidin, imaged at 10x and assessed using ImageJ: (a) adult tenocyte on an untreated coverslip, (b) adult tenocyte on a coverslip adsorbed in PBS, (c) adult on a coverslip adsorbed in FBS, (d) foetal tenocyte on an untreated coverslip, (e) foetal tenocyte on a coverslip adsorbed in PBS, (f) foetal tenocyte on a coverslip adsorbed in FBS. Scale bar represents 50µm.

When comparing the response of both adult and foetal tenocytes under serum free media and typical cell culture conditions (when serum is present in the media); it is possible to visualise how the cells reacted to the various conditions (Fig. 5.8). The foetal tenocytes overall showed they did respond to serum being presented on the surface of a substrate after at least 24 hours (Fig. 5.8a); with a significant change in both their shape and size to reflect the phenotype of foetal tenocytes under normal cell conditions after 24 hours.

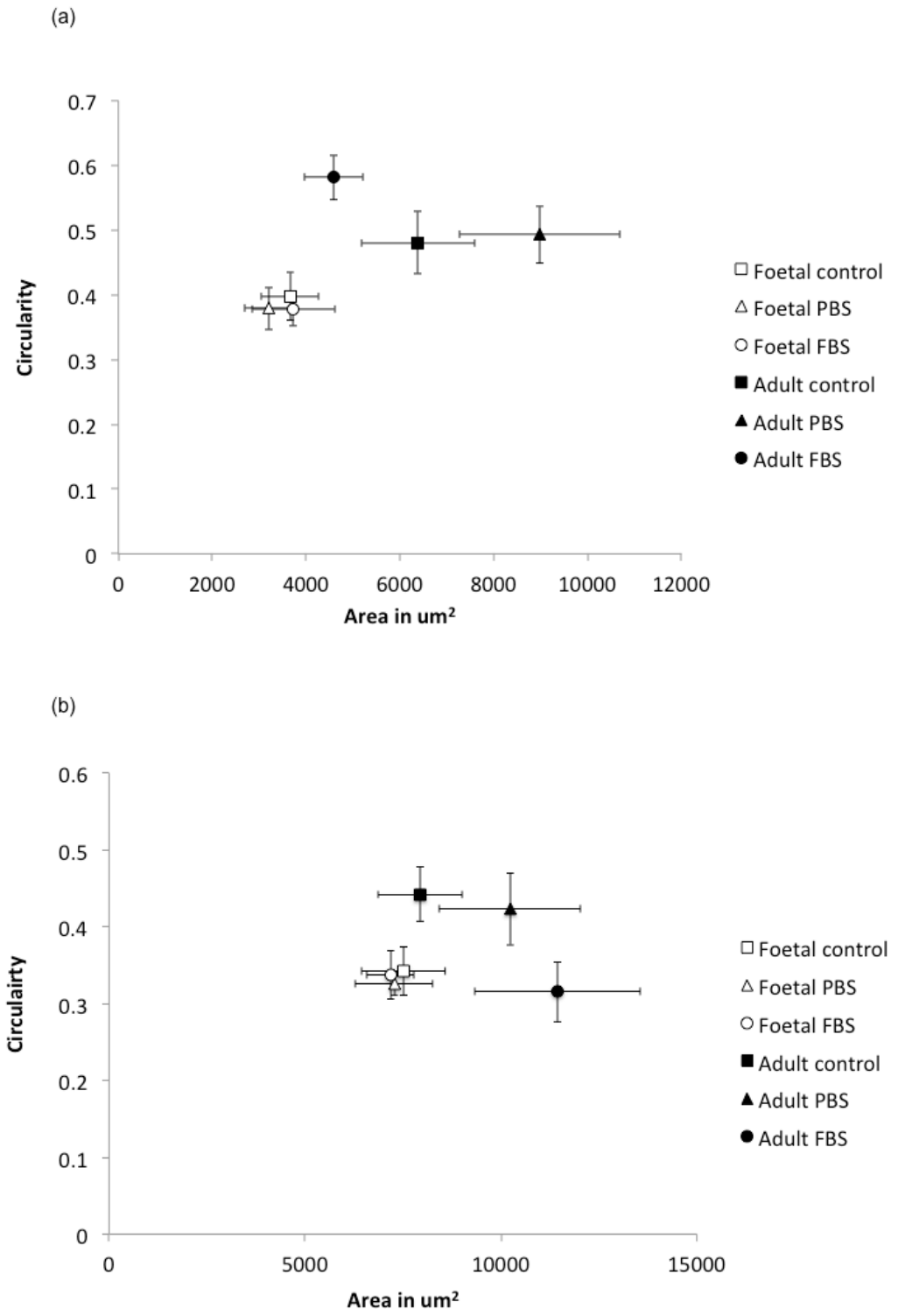


Figure 5.4 Scatter plots comparing cell morphology of adult and foetal tenocytes
Quantitative data was obtained on cell surface area and circularity through analysis of the immunostaining images using ImageJ (a) at 6 hours incubation, (b) at 24 hours incubation (n=3 N=24) Error bars are ±SEM.

The changing behaviour of adult tenocytes to the different testing conditions was less unambiguous (Fig. 5.8b). Although, there are still some interesting

observations to be made, including that after 6 hours of culture in both serum free media and normal culture media, the adult tenocytes morphology remained unchanged when serum was present on the surface of the substrate. Despite this, serum-containing media was enough to alter the cells to become more elongated in shape and larger in size after 6 hours under control conditions and when PBS was coated on the surface of the substrate.

Presenting the serum on the substrate for 24 hours to the cells in serum free media resulted in a shift in the shape of the adult tenocytes as they became more elongated than under control conditions. However there was no significant change in the size of the cells. Having serum-containing media gave rise to an alteration in morphology with the cells becoming progressively larger and less rounded; although only the size of the adult tenocytes was considerably modified.

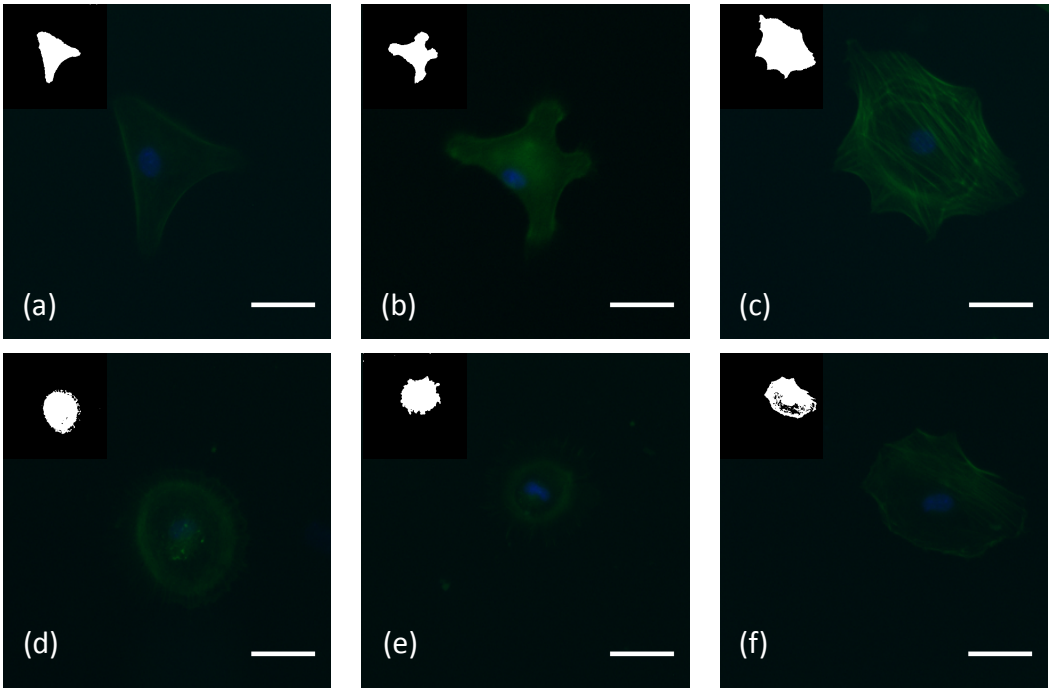


Figure 5.5 Tenocyte cell morphology after 6hrs attachment of actin cytoskeleton (green) and cell nucleus (blue) at 10x magnification with cell outline inserts.
Adult and foetal tenocytes were seeded onto glass coverslips at 5,000 cells/cm^2 in serum free media, then fixed, permeabilised and immunostained with FITC-phalloidin, imaged at 10x and assessed using ImageJ: (a) adult tenocyte on a untreated coverslip, (b) adult tenocyte on a coverslip adsorbed in PBS, (c) adult on a coverslip adsorbed in FBS, (d) foetal tenocyte on a untreated coverslip, (e) foetal tenocyte on a coverslip

adsorbed in PBS, (f) foetal tenocyte on a coverslip adsorbed in FBS. Scale bar represents 50 µm.

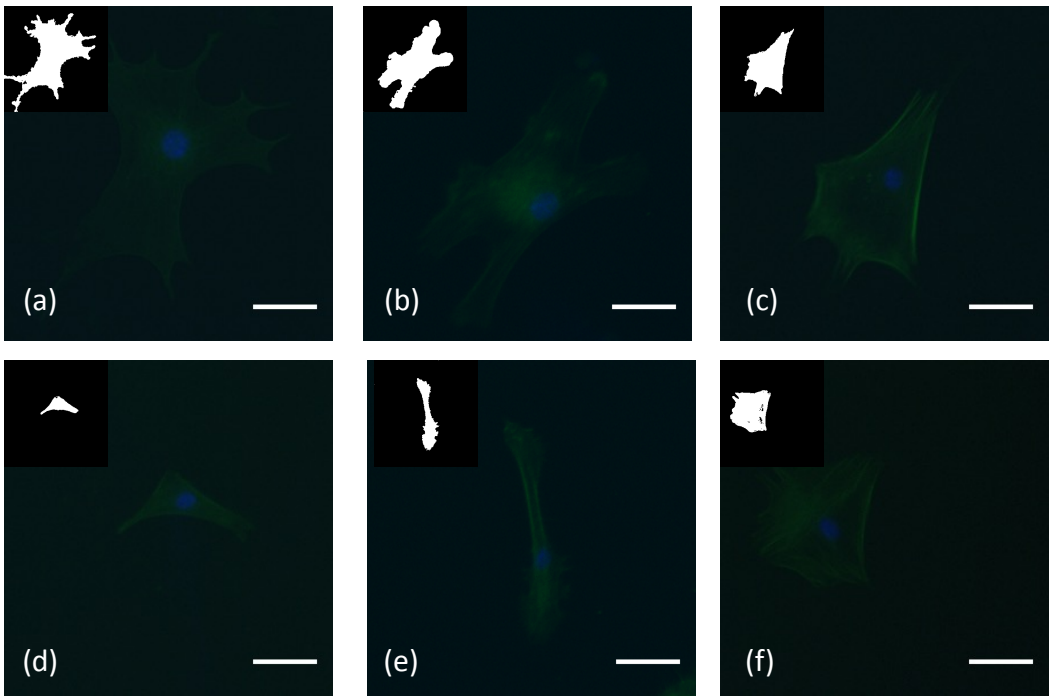


Figure 5.6 Tenocyte cell morphology after 24hrs attachment of actin cytoskeleton (green) and cell nucleus (blue) at 10x magnification with cell outline inserts.
Adult and foetal tenocytes were seeded onto glass coverslips at 5,000 cells/cm^2 in serum free media, then fixed, permeabilised and immunostained with FITC-phalloidin, imaged at 10x and assessed using ImageJ: (a) adult tenocyte on a untreated coverslip, (b) adult tenocyte on a coverslip adsorbed in PBS, (c) adult on a coverslip adsorbed in FBS, (d) foetal tenocyte on a untreated coverslip, (e) foetal tenocyte on a coverslip adsorbed in PBS, (f) foetal tenocyte on a coverslip adsorbed in FBS. Scale bar represents 50 µm.

5.2.3 Age-related tenocyte response to shear stress

Critical shear is the level of shear stress required to detach all attached cells to a substrate (Ming et al., 1998), although a 50% detachment level of cells has also been used as an endpoint (Engler et al., 2009). The results (Fig. 5.9a and 5.9b) show that for both adult and foetal tenocytes, cell attachment did not fall below 50% across a range of shear stress of 0.27 dyne/cm^2 to 1.02 dyne/cm^2 (Mackley et al., 2006). Overall, there is little evidence that the foetal tenocytes were affected within this shear stress range (Fig. 5.9a) on average, cell attachment never fell below 75%.

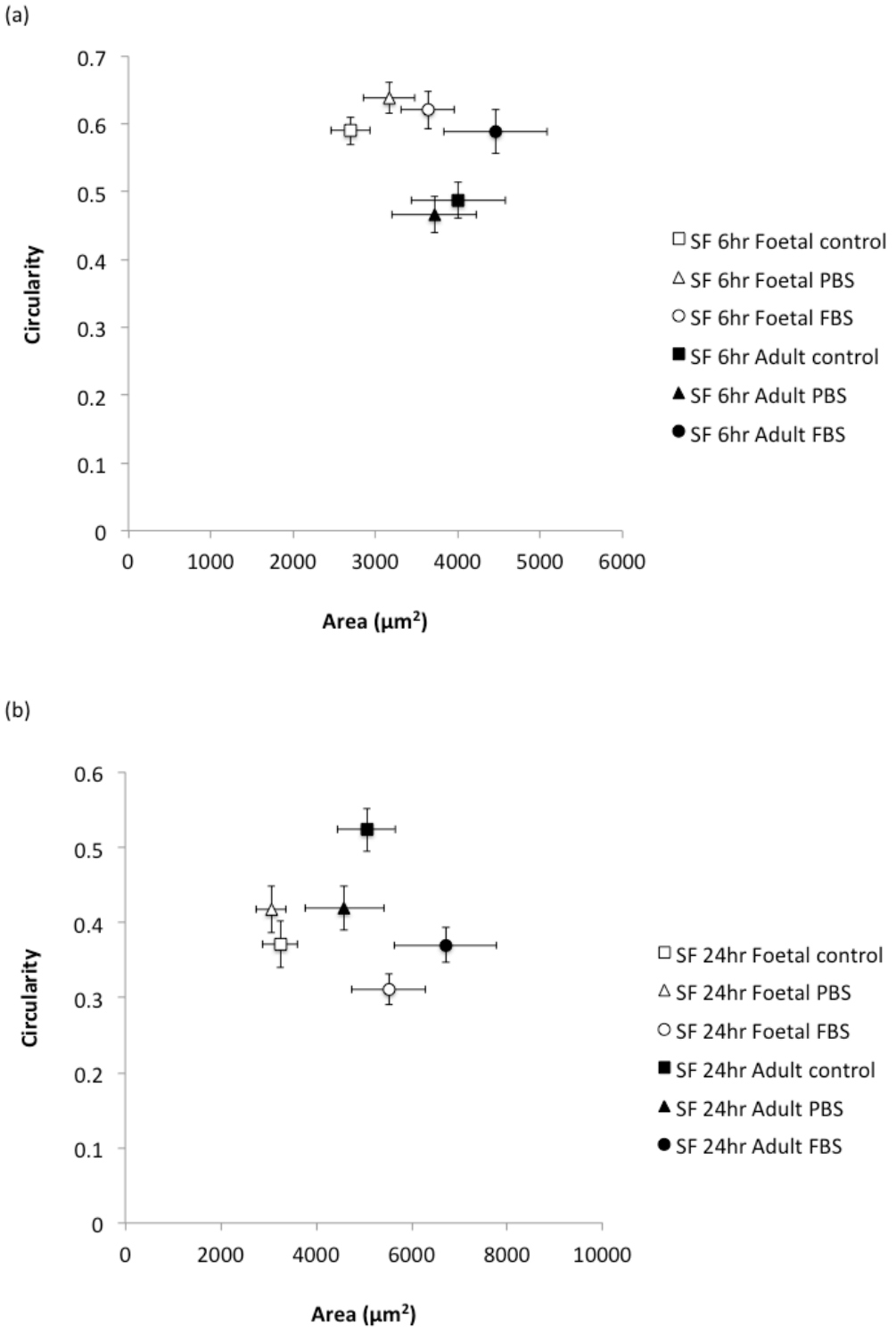


Figure 5.7 Scatter plots comparing cell morphologies of adult and foetal tenocytes (with serum free media)

Quantitative data was obtained on cell surface area and circularity through analysis of the immunostaining images using ImageJ (a) at 6 hours incubation; (b) at 24 hours incubation (n = 3 N =24) Error bars are ±SEM.

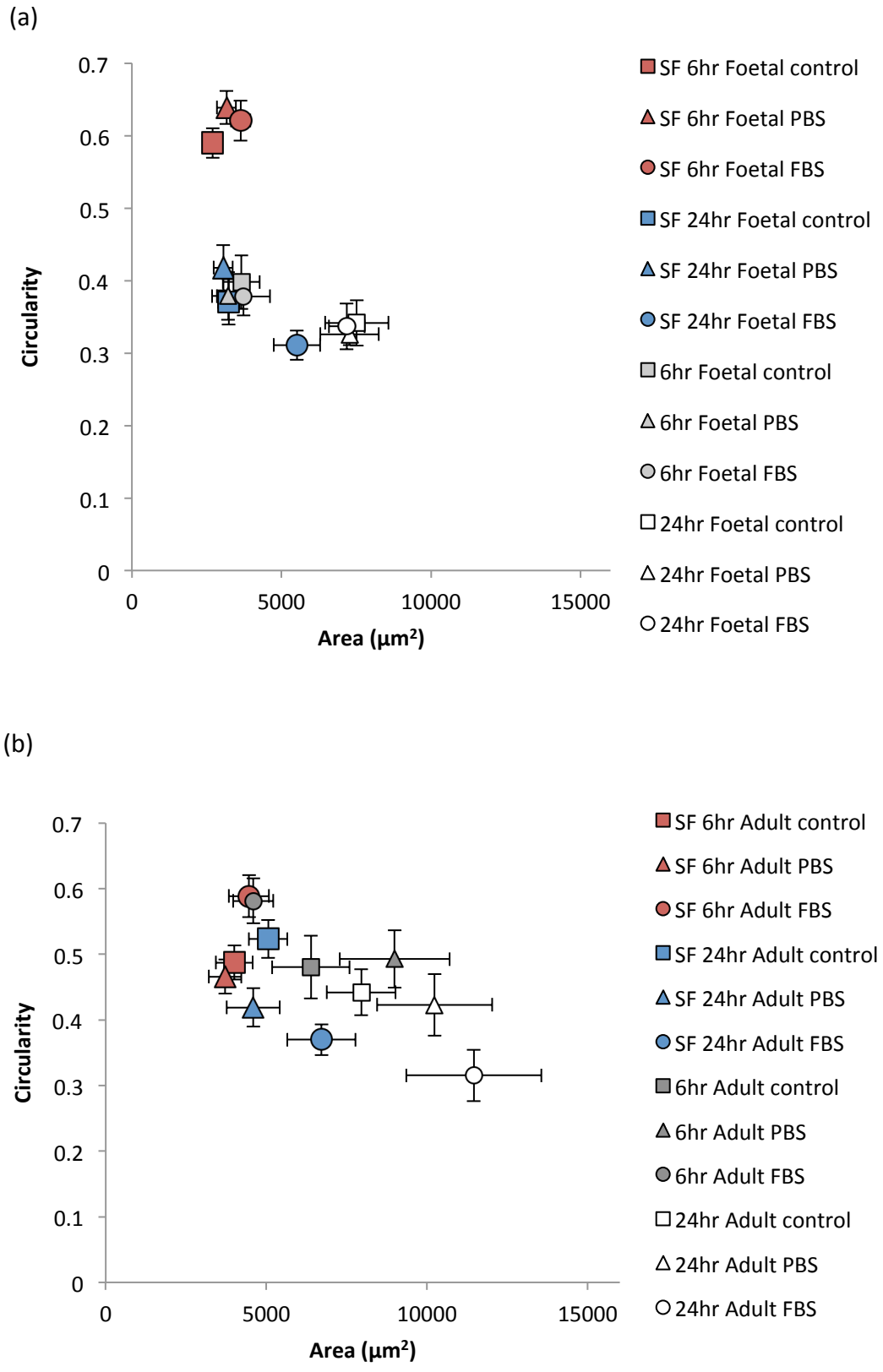


Figure 5.8 Scatter plots comparing cell morphologies of adult and foetal tenocytes with serum-containing media and serum free media
(a) at 6 hours incubation; (b) at 24 hours incubation (n = 3 N =24) Error bars are ±SEM.

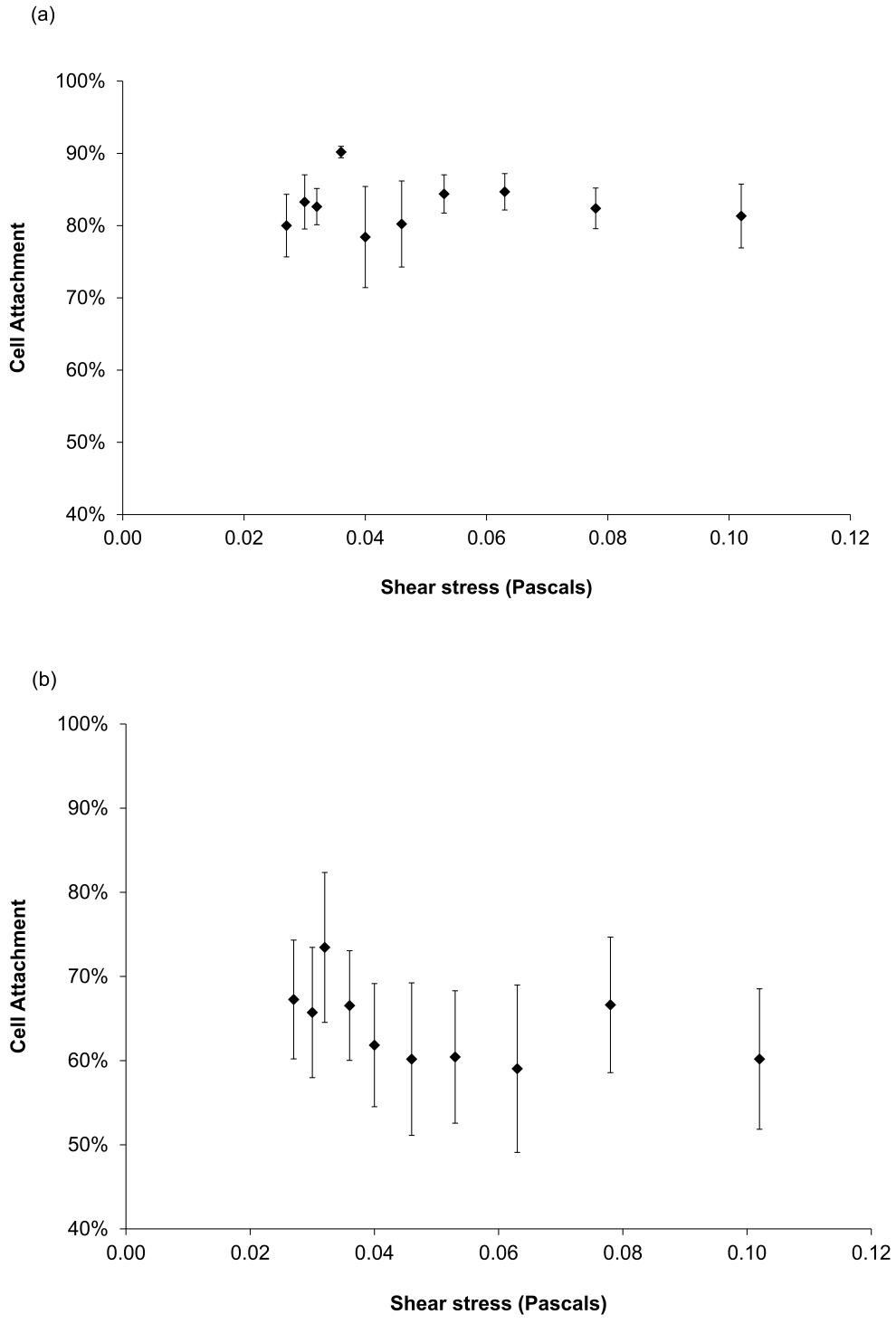


Figure 5.9 Rate of cell attachment in relation to varying levels of shear stress 0.102-0.027 Pa.

Tenocytes were seeded into the flow chamber at 10,000 cells/cm^2 and incubated at 37^{o}C and 5% CO_2 for 3 hours. After which, the cells were exposed to a preliminary wash in culture medium at a flow rate of 12ml/minute via a peristaltic pump (model) for 1 cycle of the pump. Following the wash, 30 images in 3 lines of 10 were taken from the narrow exit end of the chamber to the widest point, at 5mm intervals. (a) Foetal tenocytes (b) Adult tenocytes (n=6 N=30). Error bars are ±SEM.

In relation to the adult tenocytes, (Fig. 5.9b) there is a slight trend with larger deviation in the levels of cell attachment and on average had fewer cells attached at each point across the flow chamber in comparison to the foetal tenocytes. This would indicate the adult tenocytes display a higher sensitivity to shear stress than the foetal tenocytes, despite this, the point of critical shear was not reached with either adult or foetal tenocytes (Fig. 5.10), which is indicated as being at 3.6 dyne/cm^2 with foetal tenocytes and 2.55 dyne/cm^2 for adult tenocytes.

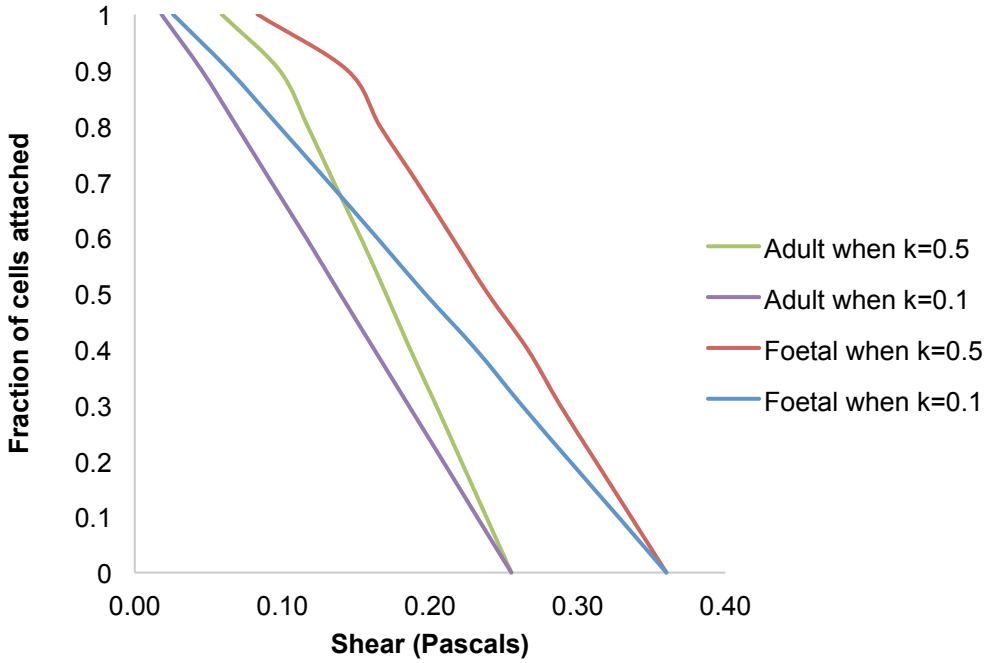


Figure 5.10 Cell detachment in relation to point of critical shear for adult and foetal tenocytes when k=0.1 and k=0.5
The theoretical critical shear was extrapolated in accordance with a formula previously devised (Ming et al., 1998) and reflects the value of shear stress at which all cells are detached from a substrate surface. The critical shear value for foetal tenocytes was 3.6 dyne/cm^2 and 2.55 dyne/cm^2 for adult tenocytes.

5.3 Discussion

The aim of the work in this chapter was to investigate and characterise adult and foetal tenocytes. In particular to demonstrate if age related differences were displayed in cell attachment, cell morphology and their cytoskeleton in response to substrate material properties and protein adsorption. This is reflective of the previous findings of others in relation to the behaviour of foetal

and adult dermal fibroblasts (Brink et al., 2009; Bullard et al., 2003; Sandulache et al., 2007)

5.3.1 Influence of serum on attachment and spreading of tenocytes

Serum-containing media

Certain aspects of the tenocyte phenotype were characterised, including the actin cytoskeleton. This is key to the response of tenocytes to mechanotransduction, which are known to be mechanically sensitive cells (Wall et al., 2007; Wang, 2006). The results demonstrate that foetal tenocytes remain remarkably unchanged in their phenotype under different conditions, even in the presence of added serum. They displayed the 'spindle like' morphology typical of tenocytes; this is evident in the first few hours of attachment and spreading.

Previous findings (Sandulache et al., 2007) demonstrated that foetal (dermal) fibroblasts exhibit a 'robust' phenotype that is relatively unaffected by external factors, including being seeded on a 2D substrate versus a 3D substrate. Thus showing they are able to withstand changes to their environment and merits further exploration of the foetal fibroblast phenotype, this could be due to cell receptors that are specific to foetal fibroblasts (Stalling and Nicoll, 2008). It was reported that foetal dermal fibroblasts promptly upregulated integrin receptors for conventional ECM proteins such as fibronectin (Cass et al., 1998).

However, the same research group showed that in low (soluble) serum conditions, foetal fibroblasts spread out to a greater extent than adult fibroblasts. This is the reverse of their behaviour in high serum conditions. Similarly, in low serum conditions, the adult fibroblasts were less spread out in terms of shape, less well attached and also produced less collagen. They cultured cells using media with varying levels of serum and found that serum in media had the opposite effect on adult and foetal fibroblasts as to the effect of serum being coated on a substrate. They showed that, in low serum conditions (with the serum in solution), the foetal tenocytes were more spread and had a significantly larger surface area than adult fibroblasts. This is in contrast with

the findings, presented here, which showed that on a serum coated substrate; it was the adult fibroblasts that displayed a significantly larger surface area than the foetal tenocytes after 24 hours culture and the foetal tenocytes showed very little variation in their phenotype across the different conditions.

Serum free media

The level of serum and the way it is presented to the cells has a significant influence on their subsequent behaviour (Allen et al., 2006). Others have shown that the way serum is offered to cells can alter the differentiation of neural stem cells (Hung and Young, 2006). If serum is present in culture medium, then the first contact that occurs, is whilst the cells are still in suspension; serum coated on a substrate will only interact with the cells once they start to attach and spread. They ascertained that cells initially reacted to the soluble form of serum before reaching the surface of the substrate.

To further investigate this, the influence of culturing the adult and foetal tenocytes in serum free media under the same previous conditions was examined. For both adult and foetal tenocytes the most substantial alteration in morphology was observed when the cells had been cultured in serum free media but had attached and spread on the serum coated substrate. This is particularly interesting in the case of the foetal tenocytes, as presenting a serum coated surface to the cells had almost the equivalent result on their morphology to when the foetal tenocytes would have been subjected to the soluble form of serum proteins in normal culture conditions.

Therefore, it could be reasonable to assume that in the case of normal culture conditions with soluble serum present, the cell surface receptors of foetal tenocytes bonded with the proteins from the serum-containing media. If the cell receptors that are unique to foetal fibroblasts (Stalling and Nicoll, 2008) could be rapidly upregulated, in the presence of soluble serum proteins, and then subsequently become blocked, thus preventing interaction between the serum proteins present on the substrate surface. Subsequently, this could

explain why the foetal tenocyte morphology was unchanged under the 3 conditions.

The adult and foetal tenocytes displayed different morphological responses in relation to how serum was presented, with the adult tenocytes reacting strongly to serum being coated on the coverslips. This would correlate with prior findings that have shown adsorption of serum proteins actively encourages cell adhesion and spreading (Allen et al., 2006; García and Boettiger, 1999). Serum free media has been used for cell culture in previous studies as proteins within the serum that settle on a substrate surface and bind together with cell membrane receptors may influence cell adhesion and cell behaviour (Das and Zouani, 2014).

Age related differences in morphology have formerly been observed between aged and young stem/progenitor cells from tendon (Kohler et al., 2013), with the aged cells being larger in size and more disperse in shape. Whereas the young cells presented themselves as being smaller in size and displayed the characteristic 'spindle-shape' associated with fibroblasts.

These findings correlate with the morphology results from the adult and foetal tenocytes above. The rationale given (Kohler et al., 2013; Labat-Robert, 2004) for this contrast in morphology was due to the influence of the actin cytoskeleton, as within aging cells the actions of actin cytoskeleton are disrupted. This means the actin fibre formation and cell matrix interactions are negatively impacted in the aged stem/progenitor cells. Cell shape in particular is heavily guided by the cytoskeleton (Rottner and Stradal, 2011). Furthermore, age related distinctions in the expression of genes responsible for moderating mechanisms such as cell adhesion and the cytoskeleton were detected between the aged and young stem/progenitor cells. Again these results would support the similar age related differences observed between the adult and foetal tenocyte morphology and cell adhesion behaviour.

5.3.2 Influence of substrate wettability and shear stress on cell adhesion and spreading

The data reflects the serum and PBS coatings also increased the hydrophilicity of the coverslips, further supports the behaviour of the adult tenocytes as they spread out (Oliveira et al., 2014): once the cell surface receptors of the adult tenocytes began interacting with serum proteins, they would have formed focal adhesions on the substrate surface causing a change in components of the cytoskeleton such as actin and ended with an alteration in cell morphology i.e. a loss of the characteristic 'spindle-like' morphology, which the foetal tenocytes succeeded in maintaining.

In vivo fluid shear results in both distortion of cell shape and fluid transfer, occurring perpendicular and in parallel to the surface of the tendon tissue (Maeda et al., 2013). The exact value of shear stress that tenocytes are exposed to *in vivo* is still unknown (Tucker et al., 2014). The influence of shear on tenocytes has been previously reported, with the studies subjecting them to shear varying from around 0.14 dyne cm^2 (Maeda et al., 2011), to between 0.029-0.068 Pa (0.29-0.68 dyne/cm^2) (Tucker et al., 2014) for human tenocytes. Tenocytes have shown themselves to be resistant to similar levels of shear stress as those used in this study (Mackley et al., 2006). Although in this particular case the tenocytes were allowed to adhere to the substrates used for 7 hours, before they were subjected to 14 hours of laminar flow at 0.1dyne/cm^2.

Furthermore, it is worth noting that the studies cited both used different apparatus and different cell types to this study. Using a convergent flow chamber provides a method to assess the response of both adult and foetal tenocytes to a range of fluid-applied shear stress by measuring the rate of cell detachment. In this present study, there were limitations to the flow chamber system that was used, as the critical shear i.e. the level of shear stress required to detach all adherent cells seeded onto a substrate (Ming et al., 1998), was clearly not reached with either the adult or foetal tenocytes, as the chamber could only sustain a flow rate within a certain range.

Similar to their relatively fixed cell morphology, the foetal tenocytes exhibited greater adhesion strength than the adult tenocytes, with the level of attachment remaining reasonably static even at higher rates of shear. If foetal tenocytes are capable of stronger cell adhesion then this supports prior studies that demonstrated that foetal fibroblasts displayed faster cell migration than adult fibroblasts (Brink et al., 2009; Kohler et al., 2013; Stalling and Nicoll, 2008). This could be attributed to the intermediate fibres in the tenocytes being more pronounced, as stronger intermediate stress fibres within the cytoskeleton imply stronger adhesion strength, as these are the fibres within the cytoskeleton responsible for the mechanical strength of cells and oppose shear stress (Alberts et al., 2002; McCue et al., 2004). However, this is contrast to prior findings that indicate that aged cells have stronger actin fibres than young cells (Kohler et al., 2013). This could indicate a disparity in the development of actin stress fibres between foetal tenocytes and mature tenocytes.

Organised actin stress fibres have been demonstrated in chicken tenocytes *in vivo* to link cells together, through the main line of strain experienced by the cells (Ralphs et al., 2002). Furthermore, stiffer 2D substrates such as tissue culture plastic and glass, will promote the development of stronger actin stress fibres within the cells, so prominent stress fibres are to be expected with cells on stiff substrates as they spread out (Tojkander et al., 2012). This was the behaviour seen with the adult tenocytes, but not with the foetal tenocytes.

This particular behaviour of the foetal tenocytes could aid in understanding how they are able to induce a reparative healing process of tendon injuries. Furthermore, if foetal dermal fibroblasts can demonstrate the same phenotype on a 3D substrate, this could imply that foetal tenocytes are capable of also doing this, making them more suited as the choice of cells for a cell delivery scaffold. Therefore, it will be important to investigate the response of adult and foetal tenocytes to shear stress on softer substrates and within a 3D environment.

5.4 Conclusion

Age related morphological differences between adult and foetal tenocytes have been demonstrated in these studies. Unlike adult tenocytes, foetal tenocytes were able to retain their morphology despite changes in substrate properties such as protein adsorption and wettability. However, there is a clear link between cell morphology and the way serum proteins are presented to the cells. The ability of foetal tenocytes to maintain this behaviour despite environmental influences could help to explain the reasons why foetal tendon undergoes regenerative healing. Additionally, foetal and adult tenocytes displayed disparities in the strength of their cell adhesion in response to shear. Ultimately, the results demonstrate that foetal tenocytes could be a suitable cell type for use in tissue engineering applications, including *in vitro* cell expansion techniques.

Chapter 6 – Foetal and mature tenocyte culture in a PLGA hollow fibre bioreactor

6.1 Introduction

PLGA has become a popular biomaterial for use in regenerative medicine applications. This is largely due to the many desirable properties it has which make it an ideal choice as a biomaterial (Gentile et al., 2014). PLGA has FDA approval for clinical use, along with non-toxic by-products from its degradation, variable elastic modulus and a porous material. For potential delivery of tenocytes to the injury site, the biomaterial would only be needed for a relatively short length of time, which is a design feature of PLGA with the correct formulation. Seeding the tenocytes on a biodegradable material like PLGA with a relatively rapid degradation rate means that it can be used as a vector to deliver cells to the injury site and then as the polymer degrades, the cells can develop their own ECM to replace it.

There has been little to no work done to comparatively investigate the behaviour of mature and foetal tenocytes on scaffolds. The aim of the work in this chapter was to assess the response of the tenocytes when expanding them under static culture conditions within a hollow fibre bioreactor (HFB) using PLGA. The response of the cells was evaluated in relation to attachment, proliferation and metabolic activity.

6.1.1 Hollow Fibre Bioreactor (HFB)

Hollow fibre bioreactors (HFB) have been used extensively for tissue engineering applications, including cell expansion (Wung et al., 2014). There are three main mechanisms for cell attachment and growth within a HFB that have been previously utilised: cells growing whilst suspended in a gel within the extracapillary space, cell attachment on the external surface of the fibres or cells seeded within the lumen of the fibres (Wung et al., 2014).

The reactor can accommodate bundles of PLGA fibres; these can range in fibre diameter size. The average diameter of fibres used in this study was 957.5 µm, this allowed for two fibres per reactor. The reactor had two external ports, which allowed media to be fed through, and could be closed off when needed. Using SEM the porosity of the fibres could be confirmed; this characterisation suggested that the mass transfer of nutrients from the media should have passed through the fibres to the cells attached inside the fibres. By seeding tenocytes inside the fibres, potential issues of removing the cells through handling the fibres could be avoided. The HFB was set up as previously described in Figures 3.2 and 3.3 in Chapter 3 – Materials and Methods.

6.2 Results

6.2.1 PLGA characterisation

Scanning electron microscopy

After preparing the PLGA fibre, scanning electron microscopy (SEM) was used to confirm the porosity of the PLGA fibres and hence that the fibres are semi-permeable allowing for diffusion of media from the extracapillary space through to the cells seeded within the fibre lumen, and vice versa for waste products produced by the cells.

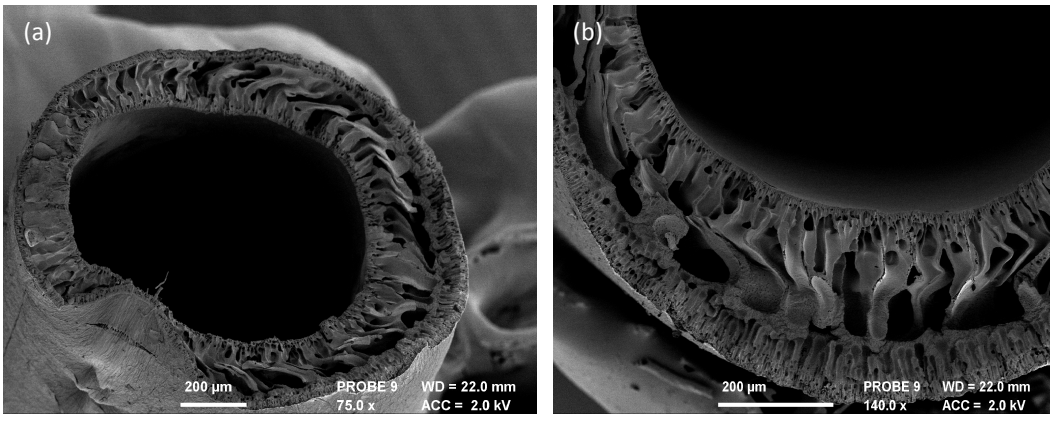


Figure 6.1 Scanning electron micrograph images of cross-sections of PLGA 75:25 fibre lumen and porosity
The PLGA fibre was cut into 1cm sections and gold sputter coated, images were taken of the fibre lumen under vacuum and surface at a working distance of 20.0mm and beam accelerating voltage of 2.0 kV: (a) at 75x magnification (b) at 140x magnification. Scale bar represents 200µm, n=3.

Tensile testing

The Youngs' modulus of the PLGA fibre (Fig. 6.2) falls within the range of Youngs' modulus previously reported for human tendon between 0.143-2.31GPa (Yang et al., 2001). Therefore, this confirms that the PLGA fibre had an appropriate stiffness to be used with tenocytes either *in vitro* or *in vivo*. The fibre under tension exhibited a very short elastic region, which is reflective of native tendon tissue (Fig. 6.3), which has a high elastic modulus under tension. This action of the collagen fibrils reflects the non-linear biomechanical behaviour that tendon is known for (Bagnaninchi et al., 2007; Connizzo et al., 2013; Dunkman et al., 2013). The low stiffness allows the tissue to support movement of the joints by transferring energy from muscle to bone; in contrast the high stiffness action allows the tissue to provide strength to the surrounding joint (Connizzo et al., 2013).

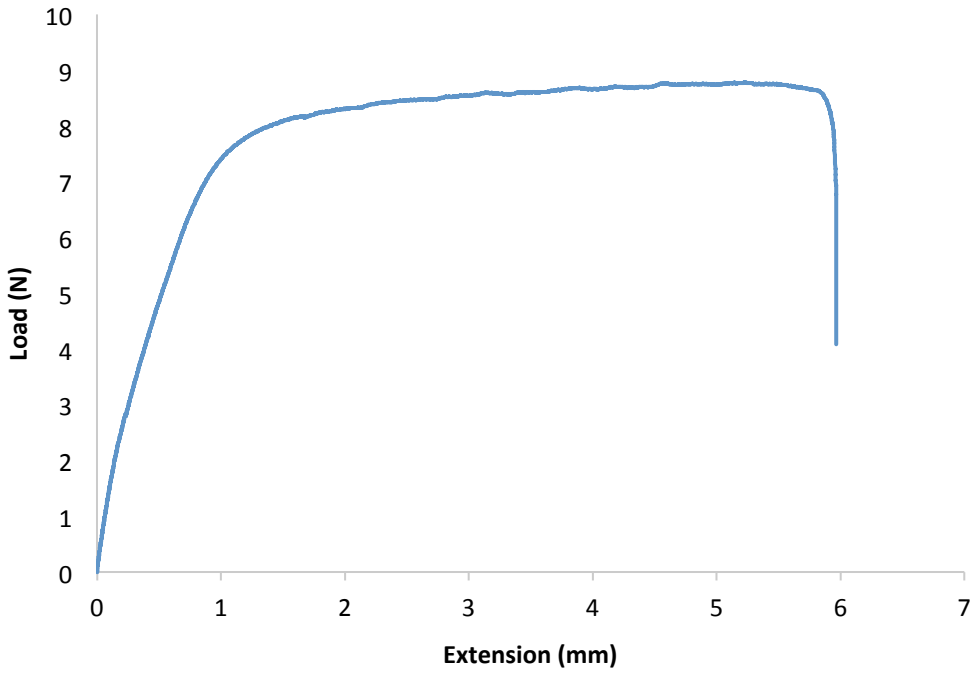


Figure 6.2 Testing of load vs. extension of PLGA 75:25 fibre
The PLGA fibre was cut into 2cm sections and loaded into an Instron 3343 machine together with a loading cell of 10N applied at a rate of 1.5mm/min. Youngs' modulus = 0.311GPa (311MPa), STDEV = ±0.07 n = 10, N = 1.

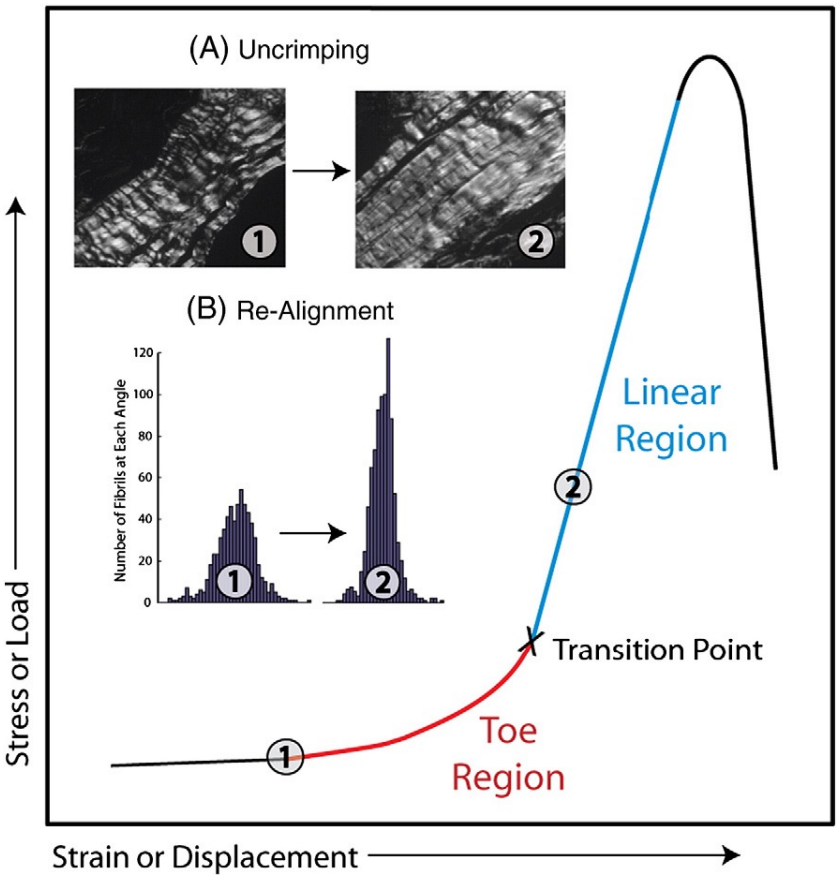


Figure 6.3 A stress-strain curve of tendon tissue
Illustrating (1) a low stiffness range within the toe region before shifting to (2) a high stiffness range within the linear region as the collagen fibrils undergo uncramping to re-aligning. Taken from (Connizzo et al., 2013).

6.2.2 Cell proliferation

A PicoGreen assay was selected as the preferred method of choice to determine cell proliferation of the tenocytes that were seeded internally in the lumen space of the PLGA hollow fibre. This particular assay has been identified as ideal for use with cells seeded within porous scaffolds (Forsey and Chaudhuri, 2009), as the assay is done on lysed cells. Furthermore, the assay is more accurate than others such as the MTT assay at low cell concentrations (Forsey and Chaudhuri, 2009). MTT is an assay that measures cell metabolism, while PicoGreen detects cell DNA. If a cell is not metabolically active then you can get a false cell number.

Mature vs. foetal cell proliferation

The final DNA content of mature (P1 mouse pups) and foetal tenocytes (E15.5) after each time point shows that there was a significant difference between the number of mature and foetal tenocytes proliferating within the HFB (Fig. 6.4). With both cell types as the length of culture increased there was a drop in the total number of cells; this trend was more noticeable with the foetal tenocytes, as there was little change in the number of mature tenocytes across the time course experiments. In contrast, the mature tenocytes showed an increase in cell population when they were cultured on TCP with lengthening culture time (Fig. 6.5). Despite this, there was no discernible trend with the foetal tenocytes on TCP, and no significant difference between the average number of mature and foetal tenocytes yielded.

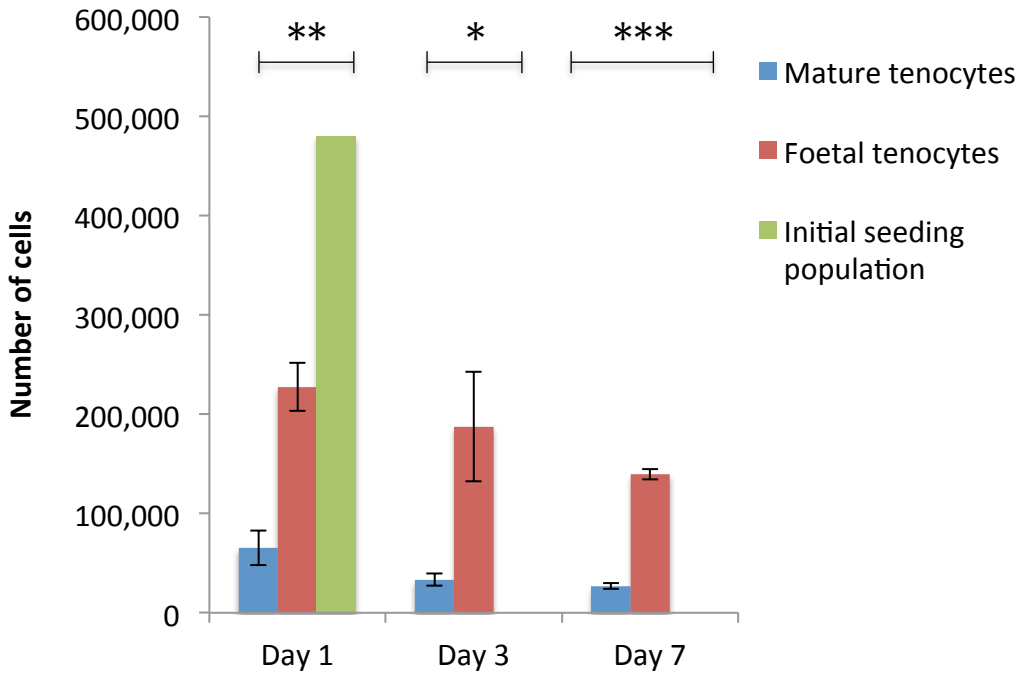


Figure 6.4 Average number of mature and foetal tenocytes cultured within the Hollow Fibre Bioreactor (HFB) at different time points
Cell attachment assays were performed by adding tenocytes at a density of 34,290 cells/cm^2 inside the lumen of the hollow fibres with 5ml of standard culture media. Only tenocytes from early passages (1 to 3) and either from P1 pups or E15.5 foetuses were used n = 3 (* = $p < 0.05$, ** = $p < 0.01$ *** = $p < 0.001$). Error bars are ± SEM.

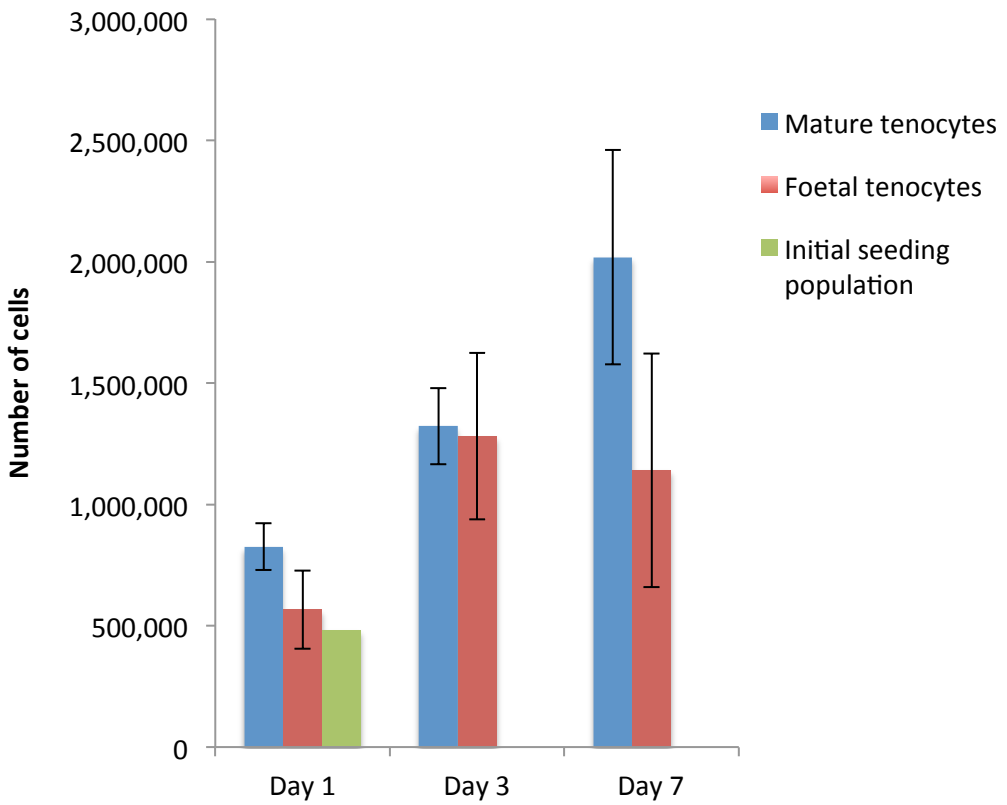
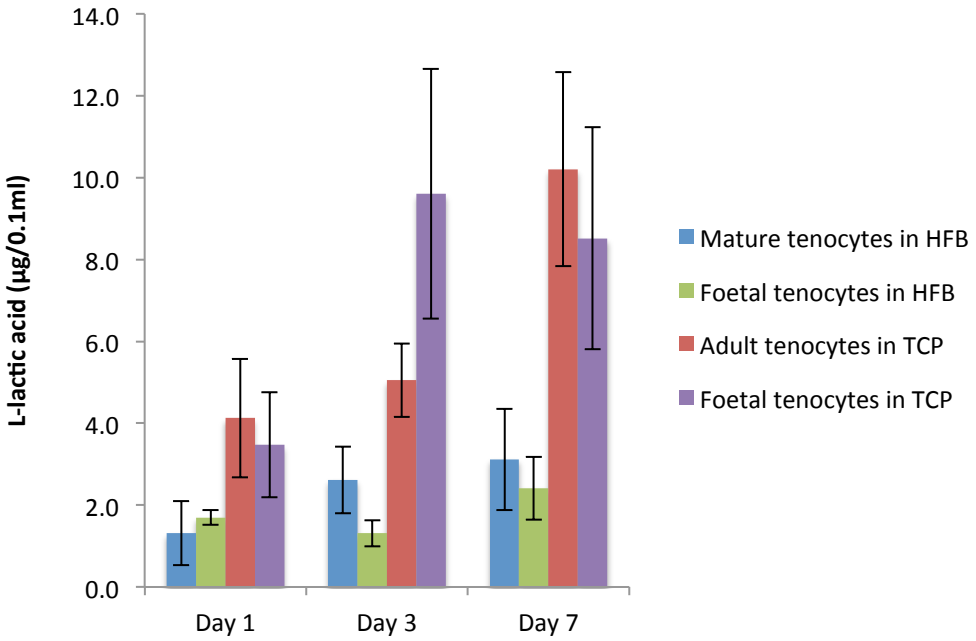


Figure 6.5 Average number of mature and foetal tenocytes cultured within the Tissue Culture Plastic (TCP) at different time points
Cell attachment assays were performed by adding tenocytes at a density of 34,290 cells/cm^2 inside T25 culture flasks with 5ml of standard culture media. Only tenocytes from early passages (1 to 3) and either from P1 pups or E15.5 foetuses were used. Cells cultured in the T25 flasks were subjected to trypsin/EDTA followed by cell counting using Trypan blue n = 3. Error bars are ± SEM.

6.2.3 Cell metabolism

To monitor the response of the cells once they had been internally seeded in the PLGA fibre lumen, the metabolism of the cells was measured, specifically in terms of glucose utilisation and lactate production. The results of lactic acid production (Fig. 6.6a) showed that there was a clear increase in the levels of waste products released by the both the mature and foetal tenocytes in the TCP with lengthening culture time. This indicates that the tenocytes preferred to proliferate on this surface to the PLGA fibres within the HFB system, where the amount of lactic acid produced by the cells remained low. Although this was to be expected with overall lower cell populations cultured in the HFB system.

(a)



(b)

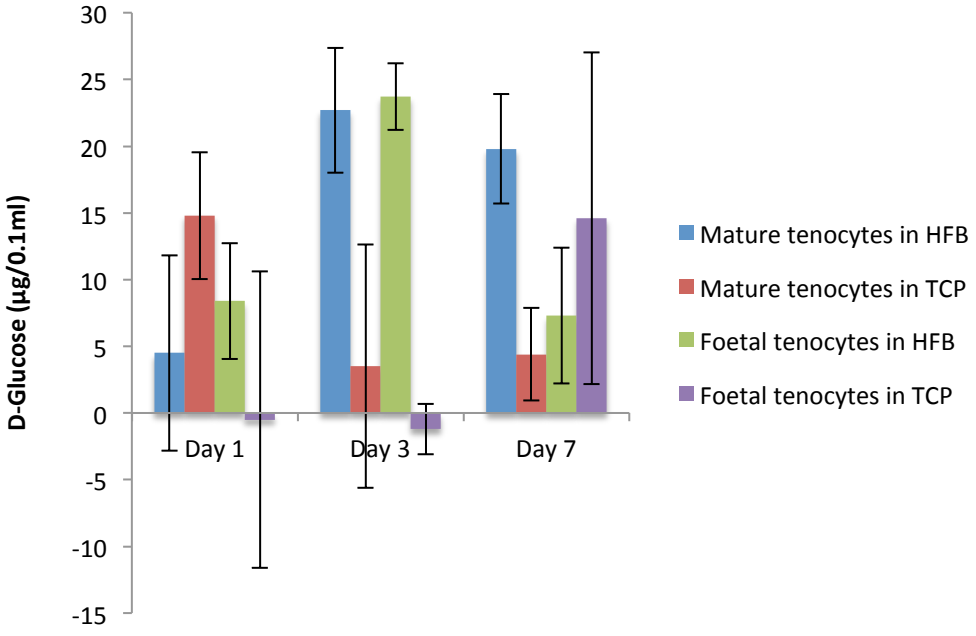


Figure 6.6 Profile of average (a) lactate formation and (b) glucose consumption for mature and foetal tenocytes with the hollow fibre bioreactor (HFB) setup and tissue culture plastic (TCP) over different time points

Media from the proliferation experiments in the HFB systems, along with media from the controls on tissue culture plastic (TCP) and within the reactors, was retained at the end of each time course experiment to be examined for the cells metabolic activity.

Within the HFB system there was an initial increase in the amount of glucose consumption (Fig. 6.6b) at Day 1, however there was a decrease in the level of glucose consumption between Day 3 and Day 7 possibly reflecting the increased presence of glucose due to an the change in culture media, rather than an increase in cell proliferation. However, by Day 7 the levels of glucose detected for the foetal tenocytes in the HFB had dropped. It is worth noting that the pattern of glucose consumption between the mature and foetal tenocytes cultured on TCP were in contrast with each other, perhaps reflecting the slower growth rates of mature tenocytes.

6.3 Discussion

6.3.1 PLGA Characterisation

Mature tendon has a distinctively high tensile strength (Marturano et al., 2013), this is needed to withstand the high tensile forces the tissue transmits from muscles to bone; however this also makes tendons vulnerable to injuries (Barber et al., 2013). The tensile strength of tendon is dependant on the thickness of tissue and the amount of collagen present and repaired tendon can see a decrease up to 30% in tensile strength compared to healthy tendon. (Sakabe and Sakai, 2011). The Youngs' modulus of human tendon is reported as between 0.143-2.31 GPa (Yang et al., 2001), the average Youngs' modulus calculated for the PLGA fibre utilised is within this range and therefore suitable for use in potential clinical applications.

The extensive network of internal pores revealed within the PLGA fibre would have enabled the culture media within the extra capillary space to perfuse through to the tenocytes seeded within the lumen of the fibres, in addition to the passage of waste products into the extracapillary space of the HFB. Moreover, the porous architecture of the fibre scaffolds is critical for initial cell attachment and in aiding cell migration during proliferation (Melchels et al., 2010). It also enables wetting by culture medium of the polymer and ultimately

should result in a high population of evenly dispersed cells grown within the fibre.

6.3.2 Cell Metabolism

The main advantage to using the HFB system under dynamic flow is that it can accommodate for an increase in cell population by maintaining a regular flow of culture media to cells whilst allowing for the removal of waste by-products such as lactate and ammonia from the cells. These waste by-products would usually slowly build up over time in traditional batch cell culture with a simultaneous decrease in vital nutrients within the culture media, thus resulting in a change in pH of the culture media, making it increasingly acidic (Tsao et al., 2005) triggering cell death and consequently limiting the maximum number of cells that can be grown in batch cell culture (Brayfield, 2008).

Despite replacing the culture media for proliferation this would have negatively impacted on the number of viable cells. Whilst it would have eliminated the waste products expelled by the cells, it would have simultaneously removed any growth factors being secreted by the cells for cell signalling. Furthermore, even though the culture media was extracted from the surrounding extracapillary space of the HFB, and the tenocytes were seeded internally within the lumen of the PLGA fibres; the removal of the media would still had the effect of potentially shearing off some cells (Rauh et al., 2014), as the tenocytes were maintained in static culture conditions within the HFB system. Excess shear stress has the effect of not only removing cells but can result in cell death of cells (Chapman et al., 2014). Previously conducted studies have indicated that tenocytes react positively to a stress of 0.14 dyne cm^{-2} (Maeda et al., 2011).

The metabolic activity of the mature and foetal tenocytes within the HFB system compared with conventional tissue culture plastic (TCP) indicates that the cells responded in contrasting ways. The foetal tenocytes produced very similar levels of lactate despite there being a significantly higher number of these cells than the mature tenocytes within the HFB's, therefore it would be expected that the accumulation of lactate would be higher for the foetal

tenocytes. This similarity could be due to the change in culture media over the full 7 day culture.

Increased glucose consumption, together with a rising levels of lactate detected in the media from the tenocytes grown on TCP reflect the cell proliferation seen across the different time point experiments (Stich et al., 2014a). In comparison, the glucose consumption of the tenocytes cultured within the HFB did not increase until after 3 days of incubation. However, it has been metabolic activity of cells is not a true reflection of cell density as a result of fluctuations within the culture environment (Tsao et al., 2005). Thus metabolic activity of should not be examined in isolation when considering the behaviour of cells.

6.3.3 Cell proliferation

The use of the PicoGreen assay avoided the need to use trypsin to detach the cells from the fibres, which would have proven difficult due to the fact the tenocytes were seeded internally in the fibres (Ellis et al., 2010). PicoGreen has a further advantage to being used to quantify cell number, in this particular instance by allowing for any cells within the pores of the fibres to be counted; as the assay can lyse the cells and release their DNA (Ellis et al., 2010). Hence previous studies have recommended the use of the PicoGreen assay for counting cells seeded either on or in scaffolds. The assay is also more accurate than others at lower cell concentrations, which is particularly useful with the mature tenocytes, where the cell numbers were consistently low.

The main disadvantage of using the assay is that it cannot distinguish between live and dead cells, therefore to determine cell viability an additional assay would have been required (Ellis et al., 2010). The PicoGreen assay does not measure cell viability directly but an increase in DNA content is a reflection that cells are proliferating (Issa et al., 2011). Therefore, the decrease in DNA content over time in the HFB suggests the tenocytes either underwent low to negligible levels of proliferation or the remaining cells were not viable. Despite the fall in DNA content measured for both the foetal and mature tenocytes, the foetal

tenocytes still displayed a significantly higher cell population than the mature tenocytes in each culturing period.

The results reflect a clear disparity firstly between the mature and foetal tendon cell populations and secondly in the type of culturing environment they were placed in. The mature tenocytes showed a steady rate of growth in cell numbers in the tissue culture plastic flasks than within the HFB. Similarly, although to a lesser extent the foetal tenocytes also had higher growth rates on the tissue culture plastic than the HFB system. Regardless of the fact the tenocytes showed greater proliferation on the tissue culture plastic there was no substantial difference in the level of growth between the two cell populations.

The high internal surface area to volume ratio of the lumen of the hollow fibres could have resulted in a high internal seeding density initially, which was not uniform within the lumen. Thus this could have negatively influenced the subsequent proliferation and metabolism of the cells (Issa et al., 2011). Furthermore, in porous scaffolds within static culture, any cells seeded too far out of reach of the edges of the lumen can experience necrosis as a result of inadequate access to nutrients, hypoxia or a combination of both factors (Melchels et al., 2010). Cell proliferation at the boundaries of the fibres lumen can prevent sufficient diffusion of nutrients and oxygen from the extracapillary space. Seeding cells statically within the HFB could have also been responsible for low seeding densities and imbalanced distribution of the cells (Melchels et al., 2010). In an attempt to mitigate this effect, equal amounts of the cell suspensions were injected within the PLGA fibres on each side of the reactors; although the extracapillary space of the HFBs were filled with DMEM whilst the cell suspensions were prepared.

Conclusions

Clearly, improvements could be made to advance the proliferation and metabolism behaviour of tenocytes to expand their growth within the HFB system using PLGA fibres. The material used for the HFB in both cases was the

same for mature and foetal tenocytes. Therefore if the material properties were the same and PLGA has been proven to be a suitable material for use with cells, its unlikely that this is the variable factor. Furthermore, the length of culture time made little difference to the increase in the number of mature tenocytes, even the foetal tenocytes experienced a drop in cell proliferation measured.

It is important that the cells for the time period they are on the material proliferate enough to successfully replace the PLGA scaffold with enough natural ECM eventually. Perfusion of the culture medium through the HFB could surmount cell necrosis due to problems with mass transport exchange of nutrients and oxygen and waste products. An additional improvement would be to incubate the HFB's in culture medium overnight ahead of the cell seeding. This would allow for enhanced cell attachment as protein adsorption from the medium onto the surface of the fibres, including the lumen.

Chapter 7 – Conclusions and Future Work

7.1 Conclusions

As neither adult nor foetal tenocytes are readily available for use in cell culture experiments, they initially required isolating from native murine tissue. This was done by testing out two frequently used cell isolation techniques of explant culture and enzyme dissociation of the tissue. Both of these methods have been previously reported as being successful in extracting tendon cells from various cell sources. Explant culture resulted in a prolonged culture time for the adult tenocytes in particular. Furthermore cell proliferation could only be measured qualitatively. Dispase and collagenase were used in conjunction or the tenocytes, to digest the tendon tissue. This had the advantage of allowing the cells to be available to be grown in culture instantly. Furthermore, this method allowed for quantitative cell proliferation, which meant that monitoring the growth of the tenocytes was much easier.

The contrasting effects between explant culture and enzyme dissociation on the adult and foetal tenocytes was clearly evidenced by the length of time it took for the adult tenocytes to begin proliferating under explant culture. The cell population of adult tendon is lower than for foetal tendon, with a higher proportion of the tissue consisting of ECM. Therefore, enzyme dissociation was selected as the best tool for tenocyte isolation as it produced the highest cell yields for both cell types whilst also being a consistent and duplicated as a method unlike explant culture.

Discernible age related differences in the morphological behaviour of adult and foetal tenocytes were highlighted in Chapter 5. Foetal tenocytes demonstrated a robust phenotype against changes to the substrate environment including the addition of proteins via serum and thus wettability. Likewise shear stress was also shown to have a more damaging influence on adult tenocytes than foetal tenocytes. Consequently, this ability of the foetal tenocytes to retain their morphological phenotype potentially mirrors the behaviour they display in vivo;

therefore aid in illuminating what is observed during the regenerative healing process following tendon injury in the foetus.

Lastly in Chapter 6 the tenocytes were seeded and cultured within a HFB using PLGA fibres. The results from the metabolism profiles and PicoGreen assays in these initial experiments indicated that the cells performed better when grown on tissue culture plastic than in the HFB setup, with more cells cultivated from the tissue culture plastic than the hollow fibre bioreactors. PLGA has been recorded as being used on multiple occasions as a successful scaffold material to proliferate cells. However, cells are typically seeded within a perfused system rather than a static culture and on the external surface of the hollow fibres instead of the internal lumen.

7.2 Future work

The main aims of this work were to successfully extract cells from native tendon tissue, characterise the phenotype of adult and foetal tenocytes and distinguish any discernible generational differences between them. This would lead to the tenocytes being seeded for expansion within a hollow fibre bioreactor with the intention of creating a feasible way of growing tendon cells rapidly in vitro for potential delivery into an injury site as a cell scaffold construct which would help to restore the tissue. Whilst it was possible to remove tenocytes from primary tissue and subsequently demonstrate that there were identifiable distinctions among the adult and foetal tenocytes; effective tenocyte culture within the HFB system using PLGA was not attained within this project. Despite this there are potential future avenues that could be explored that might make it a possibility.

7.2.1 Short-term ambitions

In the immediate future the viability of the tenocytes could be investigated within the hollow fibre bioreactor after culture with a Methylene blue assay. This would clarify whether the initial cell seeding density was appropriate or not. Furthermore it would also confirm if they reflect the results obtained on cell proliferation with the PicoGreen assay and the metabolic data on glucose

consumption and lactate production. Alternative cell viability assay that could also be considered are a calcein-AM assay or an Ethidium homodimer assay. Collating all this together would then enable optimisation of the cell seeding density within the HFB system. Additionally, to enhance the initial cell attachment of the tenocytes to the surface of the PLGA fibre lumen, the fibres could be immersed in either culture media or FBS overnight. This would encourage development of cell-protein interactions between integrins on the cell surface and the proteins found within serum.

Presently only static cultures of the tenocytes were conducted in the HFB system, there are some disadvantages to this technique that were discussed in Chapter 6. To follow on from this, perfusion culture is worth exploring. By having a steady constant flow of media through the extra capillary space of the bioreactor, the tenocytes would still be shielded from the effects of any shear stress generated by the flow of the culture media.

However, it would ensure a continuous exchange of nutrients against waste products generated by the cells as they proliferate. A perfusion culture could result in improvement of the proliferation of the tenocytes more in accordance with the way the cells grow in tissue culture plastic flasks. The length of the culture time for the tenocytes within the HFB system could also be investigated, as a longer culture time may be possible with perfused flow of media rather than a static flow.

7.2.2 Long-term ambitions

In the longer term, design of a more rational HFB system needs to be considered, for both as a cell expansion technique and as a cell delivery technique. The requirements for cell expansion would for instance necessitate that cells could be easily harvested after being expanded *in* vitro. For a cell delivery application the needs vary in that any biomaterial chosen would need to be biocompatible *in vivo* and also degrade, but at a slower rate than for cell expansion applications, where the cells would need to be available immediately. An example of such polymer materials are thermo responsive polymers, these

have been used for a wide number of purposes including gene delivery and drug delivery. These polymer materials degrade upon reaching a particular temperature.

To ensure successful cell expansion *in vitro,* the cell type utilised for future cell expansion would be an additional factor to decide upon. As this study showed, there were obvious issues with attempting to achieve effective cell proliferation with either mature or adult tenocytes, due to the initial low population of cells within the native tendon tissue. Therefore, mesenchymal stem cells (MSCs) could be used and subsequently encouraged to differentiate towards a tendon cell lineage. The differentiation of the MSCs would be tracked via assessment for tendon cell markers such as scleraxis and quantified with RT-PCR (reverse transcriptase-polymerase chain reaction). An alternative way to ensure stem cells differentiated to become tendon cells would be to use the method of co culture. This would involve culturing MSCs alongside mature tenocytes, and this technique has been tried out previously in other studies.

Further considerations that would need to be factored in when improving upon the design of the cell expansion system highlighted in this study would be to deliberate the merits both chemical and mechanical stimulation to encourage tenocyte proliferation. This includes the use of growth factors such as TGF-β (transforming growth factor-β) and exposing the cells to either cyclic tensile strain or shear stress through perfusion of culture media in the bioreactor system.

Evidently, there are clear differences to the way that adult and foetal tenocytes morphology responds to various external environmental influences. Therefore, this could have an impact which cell type is selected for expansion applications, along with other considerations for example, the effects of substrate wettability and presence or not of serum in the culture media. Lastly, if the adaptions suggested above to the current HFB system using the PLGA fibres, were investigated then there is potential that proliferation of tenocytes *in vitro* could become a viable option in future and help to develop a successful route to cell expansion applications.

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